

COMPOSITION AND METHOD FOR INHIBITING PLATELET AGGREGATION

TECHNICAL FIELD

5 This invention relates to compounds of mono and dinucleoside polyphosphates and the method of using such compounds in the prevention or treatment of diseases or conditions associated with platelet aggregation, including thrombosis in humans and other mammals.

BACKGROUND OF THE INVENTION

10 Hemostasis is the spontaneous process of stopping bleeding from damaged blood vessels. Precapillary vessels contract immediately when cut; within seconds, thrombocytes, or blood platelets, are bound to the exposed matrix of the injured vessel by a process called platelet adhesion. Platelets also stick to each other in a phenomenon known as platelet aggregation to form a platelet plug to stop bleeding quickly.

15 An intravascular thrombus results from a pathological disturbance of hemostasis. Platelet adhesion and aggregation are critical events in intravascular thrombosis. Activated under conditions of turbulent blood flow in diseased vessels or by the release of mediators from other circulating cells and damaged endothelial cells lining the vessel, platelets accumulate at a site of vessel injury and recruit further platelets into the developing thrombus. The thrombus can grow to sufficient size to block off arterial blood vessels. Thrombi can
20 also form in areas of stasis or slow blood flow in veins. Venous thrombi can easily detach portions of themselves called emboli that travel through the circulatory system and can result in blockade of other vessels, such as pulmonary arteries. Thus, arterial thrombi cause serious disease by local blockade, whereas venous thrombi do so primarily by distant blockade, or embolization. These conditions include venous thrombosis, thrombophlebitis, arterial
25 embolism, coronary and cerebral arterial thrombosis, unstable angina, myocardial infarction, stroke, cerebral embolism, kidney embolisms and pulmonary embolisms.

A number of converging pathways lead to platelet aggregation. Whatever the initial stimulus, the final common event is crosslinking of platelets by binding fibrinogen to a membrane binding site, glycoprotein IIb/IIIa (GPIIb/IIIa). Compounds that are antagonists for
30 GPIIb/IIIa receptor complex have been shown to inhibit platelet aggregation (U.S. Patent Nos. 6,037,343 and 6,040,317). Antibodies against GPIIb/IIIa have also been shown to have high antiplatelet efficacy (The EPIC investigators, *New Engl. J. Med.* (1994) 330:956-961). However, this class of antiplatelet agents sometimes causes bleeding problems.

Thrombin can produce platelet aggregation largely independently of other pathways but substantial quantities of thrombin are unlikely to be present without prior activation of platelets by other mechanisms. Thrombin inhibitors such as hirudin are highly effective antithrombotic agents. However, functioning as both antiplatelet and anti-coagulant agents, thrombin inhibitors again may produce excessive bleeding. (The TIMI 9a investigators, The GUSTO Iia investigators, *Circulation*, 90: 1624-1630 (1994); *Circulation*, 90: 1631-1637 (1994); Neuhaus K. L. *et al.*, *Circulation*, 90: 1638-1642 (1994))

Various antiplatelet agents have been studied for many years as potential targets for inhibiting thrombus formation. Some agents such as aspirin and dipyridamole have come into use as prophylactic antithrombotic agents, and others have been the subjects of clinical investigations. To date, the powerful agents such as disintegrins, and the thienopyridines ticlopidine and clopidogrel have been shown to have substantial side effects, while agents such as aspirin have useful but limited effectiveness (Hass, *et al.*, *N. Engl. J. Med.*, 321:501-507 (1989); Weber, *et al.*, *Am. J. Cardiol.* 66:1461-1468 (1990); Lekstrom and Bell, *Medicine* 70:161-177 (1991)). In particular, use of the thienopyridines in antiplatelet therapy has been shown to increase the incidence of potentially life threatening thrombotic thrombocytopenic purpura (Bennett, C.L. *et al. N. Engl. J. Med.*, (2000) 342: 1771-1777). Aspirin, which has a beneficial effect on platelet aggregation (*Br. Med. J.* (1994) 308: 81-106; 159-168), acts by inducing blockade of prostaglandin synthesis. Aspirin has no effect on ADP-induced platelet aggregation, and thus has limited effectiveness on platelet aggregation. Furthermore, its well documented high incidence of gastric side effects limits its usefulness in many patients. Clinical efficacy of some newer drugs, such as ReoPro (7E3), is impressive, but recent trials have found that these approaches are associated with an increased risk of major bleeding, sometimes necessitating blood transfusion (*New Engl. J. Med.* (1994) 330:956-961). Thus it appears that the ideal "benefit/risk" ratio has not been achieved.

Recent studies have suggested that adenosine 5'-diphosphate (ADP), a common agonist, plays a key role in the initiation and progression of arterial thrombus formation (Bernat, *et al.*, *Thromb. Haemostas.* (1993) 70:812-826); Maffrand, *et al.*, *Thromb. Haemostas.* (1988) 59:225-230; Herbert, *et al.*, *Arterioscl. Thromb.* (1993) 13:1171-1179).

ADP induces platelet aggregation, shape change, secretion, influx and intracellular mobilization of Ca^{+2} , and inhibition of adenylyl cyclase. Binding of ADP to platelet receptors is required for elicitation of the ADP-induced platelet responses. There are at least three P2

receptors expressed in human platelets: a cation channel receptor P2X₁, a G protein-coupled receptor P2Y₁, and a G protein-coupled receptor P2Y₁₂ (also referred to as P2Y_{ac} and P2_T). The P2X₁ receptor is responsible for rapid calcium influx and is activated by ATP and by ADP. However, its direct role in the process of platelet aggregation is unclear. The P2Y₁ receptor is responsible for calcium mobilization, shape change and the initiation of aggregation. P2Y₁₂ receptor is responsible for inhibition of adenylyl cyclase and is required for full aggregation. (Hourani, *et al.*, The Platelet ADP Receptors Meeting, La Thuile, Italy, March 29-31, 2000)

Ingall *et al.* (*J. Med. Chem.* 42: 213-220, (1999)) describe a dose-related inhibition of ADP-induced platelet aggregation by analogues of adenosine triphosphate (ATP), which is a weak, nonselective but competitive P2Y₁₂ receptor antagonist. Zamecnik (USPN 5,049,550) discloses a method for inhibiting platelet aggregation in a mammal by administering to said mammal a diadenosine tetraphosphate compound of App(CH₂)ppA or its analogs. Kim *et al.* (USPN 5,681,823) disclose P¹, P⁴-dithio-P², P³-monochloromethylene 5', 5''' diadenosine P¹, P⁴-tetraphosphate as an antithrombotic agent. The thienopyridines ticlopidine and clopidogrel, which are metabolized to antagonists of the platelet P2Y₁₂ receptor, are shown to inhibit platelet function *in vivo* (Quinn and Fitzgerald, *Circulation* 100:1667-1672 (1999); Geiger, *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 19:2007-2011 (1999)).

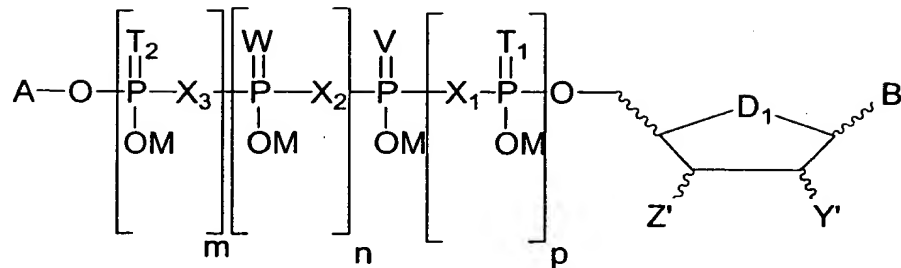
There is a need in the area of cardiovascular and cerebrovascular therapeutics for an agent that can be used in the prevention and treatment of thrombi, with minimal side effects, such as unwanted prolongation of bleeding, while preventing or treating target thrombi.

SUMMARY OF THE INVENTION

This invention is directed to a method of preventing or treating diseases or conditions associated with platelet aggregation. The method is also directed to a method of treating thrombosis. The method comprises administering to a subject a pharmaceutical composition comprising a therapeutic effective amount of P2Y₁₂ receptor antagonist compound, wherein said amount is effective to bind the P2Y₁₂ receptors on platelets and inhibit ADP-induced platelet aggregation.

The P2Y₁₂ receptor antagonist compounds useful for this invention include compounds of general Formula I, or salts thereof:

Formula I



5 wherein:

X_1 , X_2 , and X_3 are independently oxygen, methylene, monochloromethylene, dichloromethylene, monofluoromethylene, difluoromethylene, or imido;

T_1, T_2, W , and V are independently oxygen or sulfur;

$m = 0, 1$ or 2 ;

$n = 0$ or 1 ;

$p = 0, 1$, or 2 ;

where the sum of $m+n+p$ is from 1 to 5;

$M = H$ or a pharmaceutically-acceptable inorganic or organic counterion;

$D_1 = O$ or CH_2 ;

B' is a purine or a pyrimidine residue according to general Formulae IV and V which is linked to the $1'$ position of the furanose or carbocycle via the 9- or 1- position of the base, respectively;

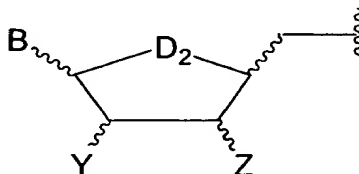
$Y' = H$, OH , or OR_1 ;

$Z' = H$, OH , or OR_2 ;

20 with the proviso that Y' and Z' are both not H , both not OH when $A=M$, and that at least one residue R_1 and R_2 is present when $A=M$;

$A = M$, or

A is a nucleoside residue which is defined as:



and which is linked to the phosphate chain via the 5' position of the furanose or carbocycle; wherein:

$D_2 = O$ or CH_2 ;

$Z = H, OH, \text{ or } OR_3$;

$Y = H, OH, \text{ or } OR_4$;

with the proviso that Z and Y are both not H, and, when Y' and Z' are H or OH, at least one residue R_3 or R_4 is present;

B is a purine or a pyrimidine residue according to general Formulae IV and V which is linked to the 1' position of the furanose or carbocycle via the 9- or 1- position of the base, respectively;

R_1, R_2, R_3 , and/or R_4 are residues which are linked directly to the 2' and /or 3' hydroxyls of the furanose or carbocycle via a carbon atom according to Formula II, or linked directly to two of the 2' and 3' hydroxyls of the furanose or carbocycle via a common carbon atom according to Formula III, such that from one to four independent residues of R_1, R_2, R_3 and R_4 falling within the definition of Formula II are present or from one to two independent residues made up of R_1+R_2 and/or R_3+R_4 are present;

The invention also provides novel pharmaceutical compositions comprising compounds of Formula Ia or Ib, which are highly selective antagonists of $P2Y_{12}$ receptor on platelets. The invention further provides a method of preventing or treating diseases or conditions associated with platelet aggregation; such diseases include venous thrombosis, thrombophlebitis, arterial embolism, coronary and cerebral arterial thrombosis, unstable angina, myocardial infarction, stroke, cerebral embolism, kidney embolisms and pulmonary embolisms.

BRIEF DESCRIPTION OF THE FIGURES

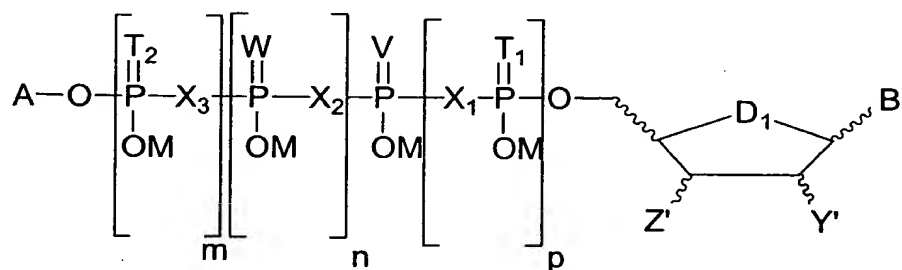
Figure 1 shows the effect of inhibition of ADP-induced aggregation by different compounds.

DETAILED DESCRIPTION OF THE INVENTION

This invention is provides a method of preventing or treating diseases or conditions associated with platelet aggregation. The method also provides a method of treating

thrombosis. The method comprises administering to a subject a pharmaceutical composition comprising a therapeutic effective amount of P2Y₁₂ receptor antagonist compound, wherein said amount is effective to bind the P2Y₁₂ receptors on platelets and inhibit ADP-induced platelet aggregation. The P2Y₁₂ receptor antagonist compounds useful for this invention include compound of general Formula I and salts thereof:

Formula I



wherein:

X₁, X₂, and X₃ are independently oxygen, methylene, monochloromethylene, dichloromethylene, monofluoromethylene, difluoromethylene, or imido;

T₁, T₂, W, and V are independently oxygen or sulfur;

m = 0, 1 or 2;

n = 0 or 1;

p = 0, 1, or 2 ;

where the sum of m+n+p is from 1 to 5;

M = H, or a pharmaceutically-acceptable inorganic or organic counterion;

D₁ = O or CH₂

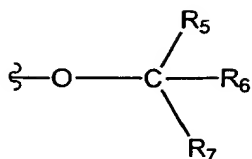
B' is a purine or a pyrimidine residue according to general Formulae IV and V which is linked to the 1' position of the furanose or carbocycle via the 9- or 1- position of the base, respectively;

Y' = H, OH, or OR₁;

Z' = H, OH, or OR₂; with the proviso that at least one of Y' and Z' is OR₁ or OR₂;

R₁ and R₂ are residues which are linked directly to the 2' and /or 3' hydroxyls of the furanose or carbocycle via a carbon atom according to Formula II, or linked directly to two of the 2' and 3' hydroxyls of the furanose or carbocycle via a common carbon atom according to Formula III,

Formula II



5 wherein:

O is the corresponding 2' and/or 3' oxygen of the furanose or carbocycle;

C is the carbon atom;

R₅, R₆, and R₇ are H, an alkyl, cycloalkyl, aralkyl, aryl, substituted aralkyl, or substituted aryl, such that the moiety defined according to Formula II is an ether; or

10 R₅ and R₆ are H, an alkyl, cycloalkyl, aralkyl, aryl, substituted aralkyl, or substituted aryl, and R₇ is alkoxy, cycloalkoxy, aralkyloxy, aryloxy, substituted aralkyloxy, or substituted aryloxy such that the moiety defined according to formula II is an acyclic acetal or ketal; or

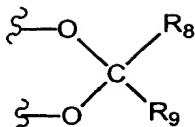
15 R₅ and R₆ are taken together as oxygen or sulfur doubly bonded to C, and R₇ is alkyl, cycloalkyl, aralkyl, aryl, substituted aralkyl, or substituted aryl, such that the moiety defined according to Formula II is an ester or thioester; or

20 R₅ and R₆ are taken together as oxygen or sulfur doubly bonded to C, and R₇ is amino or mono- or disubstituted amino, where the substituents are alkyl, cycloalkyl, aralkyl, aryl, substituted aralkyl, or substituted aryl, such that the moiety according to Formula II is a carbamate or thiocarbamate; or

25 R₅ and R₆ are taken together as oxygen or sulfur doubly bonded to C, and R₇ is alkoxy, cycloalkoxy, aralkyloxy, aryloxy, substituted aralkyloxy, or substituted aryloxy, such that the moiety according to Formula II is a carbonate or thiocarbonate; or

 R₇ is not present and R₅ and R₆ are taken together as oxygen or sulfur doubly bonded to C and both the 2' and 3' oxygens of the furanose are directly bound to C to form a cyclical carbonate or thiocarbonate;

Formula III



wherein:

O is the 2' and 3' oxygens of the furanose or carbocycle; and the 2' and 3' oxygens of the furanose or carbocycle are linked by a common carbon atom (C) to form a cyclical acetal, cyclical ketal, or cyclical orthoester;

for cyclical acetals and ketals, R₈ and R₉ are independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, substituted aralkyl, substituted aryl, or may be joined together to form a homocyclic or heterocyclic ring composed of 3 to 8 atoms, preferably 3 to 6 atoms; for cyclical orthoesters, R₈ is hydrogen, alkyl, cycloalkyl, aralkyl, aryl, substituted aralkyl, or substituted aryl, R₉ is alkyloxy, cycloalkyloxy, aralkyloxy, aryloxy, substituted aralkyloxy, or substituted aryloxy.

When present, the alkyl, cycloalkyl, aralkyl, aryl, substituted aralkyl and substituted aryl components of R₅ to R₉ may be generally defined as , but are not limited to, the following:

alkyl groups from 1 to 12 carbons, either straight chained or branched, with or without unsaturation and with or without heteroatoms, more preferably from 2 to 8 carbons, and most preferably 2 to 6 carbons;

cycloalkyl groups from 3 to 12 carbons, more preferably from 3 to 10 carbons, and most preferably 3 to 8 carbons, with or without unsaturation, and with or without heteroatoms;

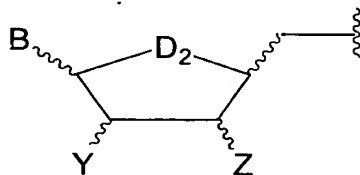
aralkyl groups from 1 to 8 carbons in the alkyl portion, more preferably from 1 to 6 carbons and most preferably 1 to 4 carbons, and are monocyclic or polycyclic moieties from 4 to 8 carbons per ring in the aryl portion , more preferably from 4 to 7 carbons, and most preferably 5 to 6 carbons per ring, with or without heteroatoms;

aryl groups, either monocyclic or polycyclic, from 4 to 8 carbons per ring, more preferably from 4 to 7 carbons, and most preferably 5 to 6 carbons per ring, with or without heteroatoms; and these groups may or may not bear substituents.

Preferred substituents on the foregoing groups may be, but are not limited to, hydroxy, nitro, methoxy, fluoro, chloro, bromo, iodo, methyl, ethyl, propyl, butyl, thioalkyl, alkoxy, carboxyl, cyano, amino, substituted amino, trifluoromethyl, phenyl, cyclopropyl, cyclopentyl, and cyclohexyl; and preferred heteroatoms are oxygen, nitrogen, and sulfur.

One embodiment of the invention is that $A = M$, wherein $M = H$ or a pharmaceutically-acceptable inorganic or organic counterion. In such embodiment, the compound is nucleoside diphosphate, nucleoside triphosphate, nucleoside tetraphosphate, nucleoside pentaphosphate, and nucleoside hexaphosphate with the 2'- and/or 3' position of the furanose or carbocycle modified. Most preferred are nucleotide diphosphates, nucleotide triphosphates, and nucleotide tetraphosphates. When T_2 , W , V , or T_1 are sulfur, the preferred position for this atom is on the terminal phosphorous of the polyphosphate chain (i.e. the phosphorous furthest removed from the nucleoside residue).

Another embodiment of the invention is that A is a nucleoside residue defined as:



and linked to the phosphate chain via the 5' position of the furanose or carbocycle (dinucleoside polyphosphate with at least one of the 2, 3, 2' and 3' position of the furanose or carbocycle modified);

wherein:

$D_2 = O$ or CH_2 ;

B is a purine or a pyrimidine residue according to general Formulae IV and V which is linked to the 1' position of the furanose or carbocycle via the 9- or 1- position of the base, respectively;

$Z = H$, OH , or OR_3 ;

$Y = H$, OH , or OR_4 ;

R_3 , and/or R_4 are residues which are linked directly to the 2' and/or 3' hydroxyls of the furanose or carbocycle via a carbon atom according to Formula II, or linked directly to

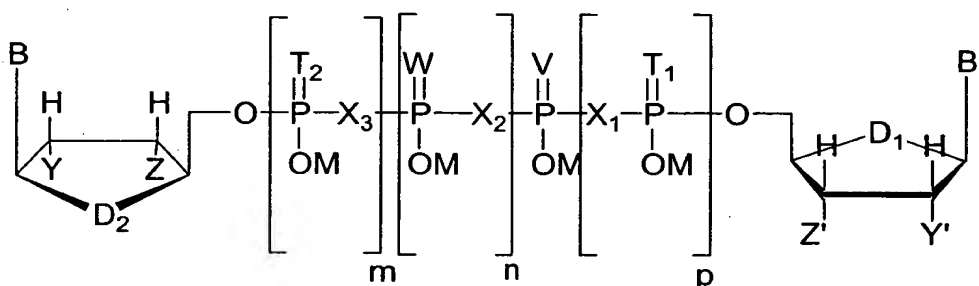
two of the 2' and 3' hydroxyls of the furanose or carbocycle via a common carbon atom according to Formula III.

When T_2 , W, V, and/or T_1 are sulfur, the preferred positions for this atom are T_1 and T_2 .

Further provisions are that when D_1 and D_2 are oxygen, the furanose is preferably in the β -configuration; and that the furanose is most preferably in the β -D-configuration.

Preferred compounds of general Formula I are molecules whose structures fall within the definitions of Formula Ia and Formula Ib:

Formula Ia



wherein:

$D_1 = O$ or CH_2 ;

$D_2 = O$ or CH_2 ;

B and B' are independently purine or pyrimidine residues according to general formula IV or V;

m and p = 0, 1 or 2; n = 0 or 1; such that the sum of m+n+p is from 1 to 5, preferably 1 to 4, and most preferably 1 to 3;

X_1 , X_2 , and X_3 = are independently O, NH, CH_2 , CHF, CHCl, CF_2 , CCl_2 ;

T_1 , T_2 , V, and W are independently O or S;

M = H^+ , NH_4^+ , Na^+ or other pharmaceutically-acceptable inorganic or organic counter ion;

$Y' = H$, OH, or OR_1 ;

$Z' = OH$ or OR_2 ;

$Z = OH$ or OR_3 ;

$Y = H$, OH, or OR_4 , where R_1 , R_2 , R_3 and R_4 falls under the definition of general formula II or III, provided that at least one of Y' , Z' , Z and Y is OR_1 , OR_2 , OR_3 , or OR_4 .

Preferred compounds of Formula Ia include:

$D_1 = O$ or CH_2 ;

$D_2 = O$ or CH_2 ;

X_1, X_2 , and $X_3 = O$;

5 T_1, T_2, V , and $W = O$; or

$D_1 = O$ or CH_2 ;

$D_2 = O$ or CH_2 ;

X_1 and $X_3 = O$;

10 $X_2 =$ methylene, monochloromethylene, dichloromethylene, monofluoromethylene, difluoromethylene, or imido;

T, T_1, T_2, V , and $W = O$; or

$D_1 = O$ or CH_2 ;

$D_2 = O$ or CH_2 ;

m, n , and $p = 1$; or

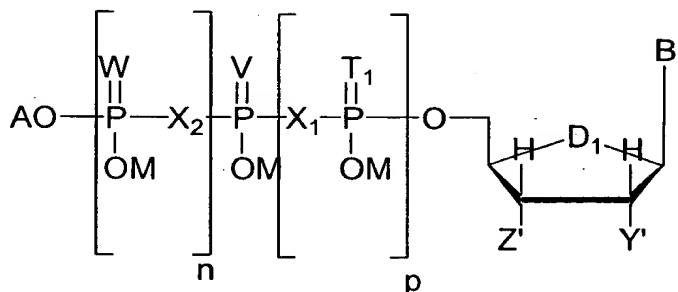
X_1 and $X_3 = O$;

$X_2 =$ methylene, monochloromethylene, dichloromethylene, monofluoromethylene, difluoromethylene, or imido;

T_1 and $T_2 = S$;

V and $W = O$.

Formula Ib



25 $D_1 = O$ or CH_2 ;

n and $p = 0, 1$, or 2 such that the sum of $n+p$ is from 0 to 3 ;

A = M; wherein M= H⁺, NH₄⁺, Na⁺ or other pharmaceutically-acceptable inorganic or organic counterion;

B' is purine or pyrimidine residue according to general Formulae IV and V;

X₁ and X₂ are independently O, NH, CH₂, CHF, CHCl, CF₂, CCl₂;

5 T₁, V, and W are independently O or S;

Y'= H, OH, or OR₁,

Z'= H, OH or OR₂, where R₁ and R₂ fall under the definitions of general Formulae II or III; with the proviso that at least one of Y' and Z' is OR₁ or OR₂, respectively.

10 Preferred compounds of Formula Ib include:

D₁ = O or CH₂;

n and p = 0, 1, or 2 such that the sum of n+p is from 0 to 3, preferably 1 to 2;

X₁ and X₂= O;

T₁, V, and W= O; or

D₁ = O or CH₂;

X₁ and X₂= O;

T₁ and V= O;

W=S; or

D₁ = O or CH₂;

p = 0, 1, or 2 such that the sum of n+p is from 1 to 3; n=1;

X₁=O;

25 X₂= methylene, monochloromethylene, dichloromethylene, monofluoromethylene, difluoromethylene, or imido;

T₁, V, and W= O;

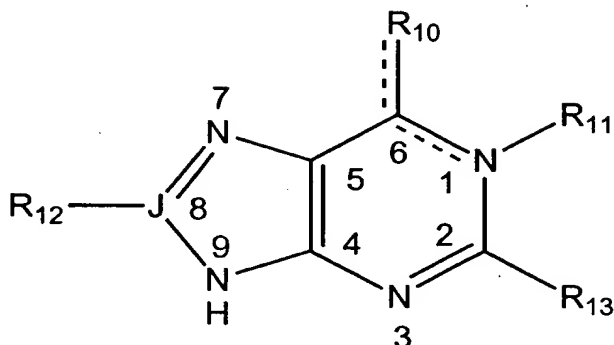
Y'= H, OH, or OR₁;

Z'= H, OH or OR₂, where R₁ and R₂ falls under the definition of general Formula II or III; with the proviso that at least one of Y' and Z' is OR₁ or OR₂, respectively.

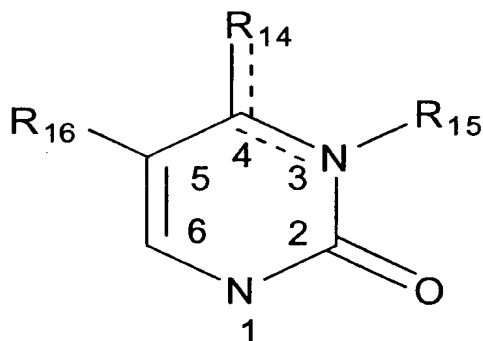
30 B and B' are independently a purine residue, as in Formula IV, linked through the 9-position, or a pyrimidine residue, as in Formula V, linked through the 1- position. The

ribosyl moieties are in the D- configuration, as shown, but may be L-, or D- and L-. The D- configuration is preferred.

Formula IV



Formula V



wherein:

- 10 R_{10} and R_{14} are hydroxy, oxo, amino, mercapto, alkylthio, alkyloxy, aryloxy, alkylamino, cycloalkylamino, aralkylamino, arylamino, diaralkylamino, diarylamino, or dialkylamino, where the alkyl groups are optionally linked to form a heterocycle; or
 R_{10} and R_{14} are acylamino, provided that they incorporate an amino residue from the C-6 position of the purine or the C-4 position of the pyrimidine; or
- 15 when R_{10} in a purine or R_{14} in a pyrimidine has as its first atom nitrogen, R_{10} and R_{11} or R_{14} and R_{15} are taken together to form a 5-membered fused imidazole ring (etheno compounds), optionally substituted on the etheno ring with alkyl, cycloalkyl, aralkyl, or aryl moieties, as described for R_5 - R_9 above;

J is carbon or nitrogen, with the provision that when nitrogen, R₁₂ is not present;
 R₁₁ is hydrogen, O (adenine 1-oxide derivatives) or is absent (adenine derivatives);
 R₁₅ is hydrogen, or acyl (e.g. acetyl, benzoyl, phenylacetyl, with or without substituents);

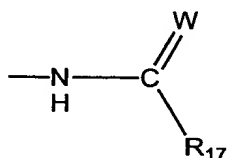
5 R₁₂ is hydrogen, alkyl, bromo, azido, alkylamino, arylamino or aralkylamino, alkoxy, aryloxy or aralkyloxy, alkylthio, arylthio or aralkylthio, or ω-A(C₁₋₆alkyl)B-, wherein A and B are independently amino, mercapto, hydroxy or carboxyl;

R₁₃ is hydrogen, chlorine, amino, monosubstituted amino, disubstituted amino, alkylthio, arylthio, or aralkylthio, where the substituent on sulfur contains up to a maximum
 10 of 20 carbon atoms, with or without unsaturation;

R₁₆ is hydrogen, methyl, alkyl, halo, alkyl, alkenyl, substituted alkenyl, alkynyl, or substituted alkynyl.

Compounds according to Formulae IV and V where R₁₀ or R₁₄ is acylamino for the most part fall within the scope of Formula VI:

Formula VI



wherein:

20 NH is the amino residue at the C-6 position in a purine or the amino residue at the C-4 position in a pyrimidine;

C is a carbon atom;

W is oxygen or sulfur;

R₁₇ is amino or mono- or disubstituted amino such that the moiety according to
 25 Formula VI is a urea or thiourea; or R₁₇ is alkoxy, aralkyloxy, aryloxy, substituted aralkyloxy, or substituted aryloxy, such that the moiety according to Formula VI is a carbamate or thiocarbamate; or

R₁₇ is alkyl, cycloalkyl, aralkyl, or aryl, with or without substituents or heteroatoms, such that the moiety according to Formula VI is an amide; with definitions of alkyl, cycloalkyl, aralkyl, or aryl groups as previously defined for comparable groups in R₅ to R₉.

The compounds of the present invention may be conveniently synthesized by those skilled in the art using well-known chemical procedures. Mononucleoside mono-, di- and triphosphates may be obtained from commercial sources or may be synthesized from the nucleoside using a variety of phosphorylation reactions which may be found in the chemical literature. Symmetrical and unsymmetrical dinucleotide polyphosphates may be prepared by activation of a nucleoside mono-, di- or triphosphate with a coupling agent such as, but not limited to, dicyclohexylcarbodiimide or 1, 1'-carbonyldiimidazole, followed by condensation with another nucleoside mono-, di-, or triphosphate, which may be the same or different as the activated moiety. Activation of nucleoside triphosphates with dicyclohexylcarbodiimide gives a cyclical trimetaphosphate as the activated species, which may be advantageously reacted with a variety of nucleophiles to install unique substituents on the terminal phosphate of a triphosphate.

The compounds of the present invention may be prepared by derivatization or substitution at the level of the nucleoside, followed by phosphorylation and condensation as previously described, or the reactions may be carried out directly on the preformed mono- or dinucleotides. In the general Formulae Ia and Ib, the substituents at Y', Z', Y, and Z may be esters, carbamates, or carbonates, which are generally described by Formula II. Esters may be readily prepared by reacting a hydroxyl group of the furanose in a nucleoside or nucleotide with an activated form of an appropriate organic acid, such as an acid halide or acid anhydride in the presence of an organic or inorganic base. Alternately, use of a suitable coupling reagent such as dicyclohexylcarbodiimide, 1,1'- carbonyldiimidazole and the like to activate the organic acid may be used to achieve the same result.

Carbamates or thiocarbamates may be most conveniently prepared by reaction of a hydroxyl group of the furanose in a nucleoside or nucleotide with any of a number of commercially available isocyanates or isothiocyanates, respectively, in an inert solvent. Alternately, when a desired isocyanate or isothiocyanate cannot be obtained from commercial sources, they may be prepared from the corresponding amine by the use of phosgene or thiophosgene, respectively, or their chemical equivalents. Carbonates or thiocarbonates may

be synthesized by reacting the hydroxyl groups of a furanose in a nucleoside or nucleotide with an appropriate haloformate in the presence of an organic or inorganic base.

In the general Formulae Ia and Ib, the substituents at Y' and Z', and Y and Z, when taken together, may be taken to mean acetals, ketals or orthoesters, as described in Formula

III. Acetals and ketals may be readily prepared by reaction of the neighboring 2' and 3' hydroxyl groups of the furanose in an appropriate nucleoside or nucleotide with an aldehyde or ketone, respectively, or their chemical equivalents, in the presence of an acid catalyst. Particularly advantageous is the use of an organic acid, which can effect the transformation without affecting the integrity of the rest of the molecule. Alternately, strong acids such as trichloroacetic, p-toluenesulfonic, methanesulfonic and the like may be employed in catalytic amounts, in conjunction with inert solvents. Most preferred is formic acid, which is ideally suited to serve as both solvent and catalyst for these reactions. The discovery of the utility of formic acid for this purpose is one particular aspect of this invention.

Cyclical orthoesters may be prepared by reaction of the neighboring 2' and 3' hydroxyl groups of a furanose with an acyclic orthoester, in the presence of an acid. When the nucleoside or nucleotide to be derivatized is a purine that contains a 6-amino functionality or is a pyrimidine that contains a 4-amino functionality, it may be converted to the respective urea or thiourea by treatment with isocyanates or isothiocyanates, respectively, as was previously described for carbamates or thiocarbamates of the 2' or 3' hydroxyls of the furanose. It was found that reactions of the amino group with isocyanates or isothiocyanates could be carried out in the presence of the hydroxyl groups of the furanose, by appropriate manipulation of the stoichiometry of the reaction.

All of the derivitization reactions described may be carried out on preformed dinucleotide polyphosphates, which results in multiple products dependent of reaction stoichiometry and on whether multiple reactive groups are present. When multiple products are obtained, these may be conveniently separated by the use of preparative reverse phase high performance liquid chromatography (HPLC). Particularly advantageous is the use of C18 or phenyl reverse phase columns, in conjunction with gradients that start with ammonium acetate buffer and end with methanol. The use of a buffer provides for nucleotide stability and improved peak shape of the eluting products and the use of methanol allows for effective desorption of these lipophilic compounds from the column. Particularly advantageous is the use of ammonium acetate buffer solutions in conjunction with methanol,

as these solvents are miscible in all proportions and may be readily removed from the chromatographed products by evaporation, followed by lyophilization.

While separation of multiple products may be done by HPLC, another strategy is to use nucleosides or nucleotides which contain only a single reactive functionality, whether because only one is present, or by the use of protecting groups to block side reactions at other positions in the molecule. This may be done at the level of preformed dinucleotide polyphosphates, or alternately, may be carried out on nucleoside mono-, di-, or triphosphates, leading to novel products in their own right, or may be coupled to other nucleoside mono-, di-, or triphosphates by the methods which have already been described.

The inventors of the present invention have discovered compounds that are antagonists of the effect of ADP on its platelet membrane receptor, the P2Y₁₂ receptor. The compounds provide efficacy as antithrombotic agents by their ability to block ADP from acting at its platelet receptor site and thus prevent platelet aggregation. Thus, these compounds can provide a more efficacious antithrombotic effect than aspirin, but with less profound effects on bleeding than antagonists of the fibrinogen receptor. Since ADP-induced platelet aggregation is mediated by the simultaneous activation of both P2Y₁₂ and P2Y₁ receptors, the combined administration of the compounds described here with antagonists of platelet P2Y₁ receptors could potentially provide a more efficacious antithrombotic effect at concentrations of each antagonist that are below the effective concentrations to block each receptor subtype in other systems, resulting in a decrease of the potential manifestation of adverse effects. In addition, these compounds can be used in conjunction with lower doses of these other agents which inhibit platelet aggregation by different mechanisms, to reduce the toxicity of these agents. Finally, if the compounds of the present invention have sufficient binding affinity and bear a fluorescent moiety, they can find uses as biochemical probes for the P2Y₁₂ receptor.

The compounds of general Formula I are useful in therapy, in particular in the prevention of platelet aggregation. The compounds of the present invention are thus useful as anti-thrombotic agents, and are thus useful in the treatment or prevention of unstable angina, coronary angioplasty (PTCA) and myocardial infarction.

The compounds of the present invention are also useful in the treatment or prevention of primary arterial thrombotic complications of atherosclerosis such as thrombotic stroke, peripheral vascular disease, myocardial infarction without thrombolysis.

Still further indications where the compounds of the invention are useful are for the treatment or prevention of arterial thrombotic complications due to interventions in atherosclerotic disease such as angioplasty, endarterectomy, stent placement, coronary and other vascular graft surgery.

5 Still further indications where the compounds of the invention are useful are for the treatment or prevention of thrombotic complications of surgical or mechanical damage such as tissue salvage following surgical or accidental trauma, reconstructive surgery including skin flaps, and "reductive" surgery such as breast reduction.

10 The compounds of the present invention are also useful for the prevention of mechanically-induced platelet activation in vivo such as cardiopulmonary bypass (prevention of microthromboembolism), prevention of mechanically-induced platelet activation in vitro such as the use of the compounds in the preservation of blood products, e.g. platelet concentrates, prevention of shunt occlusion such as renal dialysis and plasmapheresis, thrombosis secondary to vascular damage/inflammation such as vasculitis, arteritis, glomerulonephritis and organ graft rejection.

Still further indications where the compounds of the present invention are useful are indications with a diffuse thrombotic/platelet consumption component such as disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, heparin-induced thrombocytopenia and pre-eclampsia/eclampsia.

20 Still further indications where the compounds of the invention are useful are for the treatment or prevention of venous thrombosis such as deep vein thrombosis, veno-occlusive disease, hematological conditions such as thrombocythemia and polycythemia, and migraine.

In a particularly preferred embodiment of the present invention, the compounds are used in the treatment of unstable angina, coronary angioplasty and myocardial infarction.

25 In another particularly preferred embodiment of the present invention, the compounds are useful as adjunctive therapy in the prevention of coronary arterial thrombosis during the management of unstable angina, coronary angioplasty and acute myocardial infarction, i.e. perithrombolysis. Agents commonly used for adjunctive therapy in the treatment of thrombotic disorders may be used, for example heparin and/or aspirin, just to mention a few.

30 A method of treating a mammal to alleviate the pathological effects of atherosclerosis and arteriosclerosis, acute MI, chronic stable angina, unstable angina, transient ischemic attacks and strokes, peripheral vascular disease, arterial thrombosis, preeclampsia, embolism,

restenosis or abrupt closure following angioplasty, carotid endarterectomy, and anastomosis of vascular grafts.

The compounds of this invention may be used in vitro to inhibit the aggregation of platelets in blood and blood products, e.g. for storage, or for ex vivo manipulations such as in diagnostic or research use. This invention also provides a method of inhibiting platelet aggregation and clot formation in a mammal, especially a human, which comprises the internal administration of a compound of Formula (I) and a pharmaceutically acceptable carrier.

Chronic or acute states of hyper-aggregability, such as disseminated intravascular coagulation (DIC), septicemia, surgical or infectious shock, post-operative and post-partum trauma, cardiopulmonary bypass surgery, incompatible blood transfusion, abruptio placenta, thrombotic thrombocytopenic purpura (TTP), snake venom and immune diseases, are likely to be responsive to such treatment.

This invention further provides a method for inhibiting the reocclusion of an artery or vein following fibrinolytic therapy, which comprises internal administration of a compound of Formula (I) and a fibrinolytic agent. When used in the context of this invention, the term fibrinolytic agent is intended to mean any compound, whether a natural or synthetic product, which directly or indirectly causes the lysis of a fibrin clot. Plasminogen activators are a well known group of fibrinolytic agents. Useful plasminogen activators include, for example, anistreplase, urokinase (UK), pro-urokinase (pUK), streptokinase (SK), tissue plasminogen activator (tPA) and mutants, or variants thereof, which retain plasminogen activator activity, such as variants which have been chemically modified or in which one or more amino acids have been added, deleted or substituted or in which one or more functional domains have been added, deleted or altered such as by combining the active site of one plasminogen activator or fibrin binding domain of another plasminogen activator or fibrin binding molecule.

Extracorporeal circulation is routinely used for cardiovascular surgery in order to oxygenate blood. Platelets adhere to surfaces of the extracorporeal circuit. Platelets released from artificial surfaces show impaired hemostatic function. Compounds of the invention may be administered to prevent adhesion.

Other applications of these compounds include prevention of platelet thrombosis, thromboembolism and reocclusion during and after thrombolytic therapy and prevention of

platelet thrombosis, thromboembolism and reocclusion after angioplasty of coronary and other arteries and after coronary artery bypass procedures.

The compounds of the present invention also encompass their non-toxic pharmaceutically acceptable salts, such as, but not limited to, an alkali metal salt such as sodium or potassium; an alkaline earth metal salt such as manganese, magnesium or calcium; or an ammonium or tetraalkyl ammonium salt, i.e., NX_4^+ (wherein X is C_{1-4}). Pharmaceutically acceptable salts are salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects.

Those skilled in the art will recognize various synthetic methodologies which may be employed to prepare non-toxic pharmaceutically acceptable salts and acylated prodrugs of the compounds.

The active compounds may be administered systemically to target sites in a subject in need such that the extracellular concentration of a $P2Y_{12}$ agonist is elevated to block the binding of ADP to $P2Y_{12}$ receptor, thus inhibit the platelet aggregation. The term systemic as used herein includes subcutaneous injection, intravenous, intramuscular, intrasternal injection, intravitreal injection, infusion, inhalation, transdermal administration, oral administration, rectal administration and intra-operative instillation.

For systemic administration such as injection and infusion, the pharmaceutical formulation is prepared in a sterile medium. The active ingredient, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Adjuvants such as local anesthetics, preservatives and buffering agents can also be dissolved in the vehicle. The sterile indictable preparation may be a sterile indictable solution or suspension in a non-toxic acceptable diluent or solvent. Among the acceptable vehicles and solvents that may be employed are sterile water, saline solution, or Ringer's solution.

Another method of systemic administration of the active compound involves oral administration, in which pharmaceutical compositions containing active compounds are in the form of tablets, lozenges, aqueous or oily suspensions, viscous gels, chewable gums, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

For oral use, an aqueous suspension is prepared by addition of water to dispersible powders and granules with a dispersing or wetting agent, suspending agent one or more preservatives, and other excipients. Suspending agents include, for example, sodium carboxymethylcellulose, methylcellulose and sodium alginate. Dispersing or wetting agents

include naturally-occurring phosphatides, condensation products of an allylene oxide with fatty acids, condensation products of ethylene oxide with long chain aliphatic alcohols, condensation products of ethylene oxide with partial esters from fatty acids and a hexitol, and condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anydrides. Preservatives include, for example, ethyl, and n-propyl p-
5 hydroxybenzoate. Other excipients include sweetening agents (e.g., sucrose, saccharin), flavoring agents and coloring agents. Those skilled in the art will recognize the many specific excipients and wetting agents encompassed by the general description above.

For oral application, tablets are prepared by mixing the active compound with
10 nontoxic pharmaceutically acceptable excipients suitable for the manufacture of tablets. These excipients may be, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example, starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The
15 tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example,
20 calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin or olive oil. Formulation for oral use may also be presented as chewable gums by embedding the active ingredient in gums so that the active ingredient is slowly released upon chewing.

Additional means of systemic administration of the active compound to the target
25 platelets of the subject would involve a suppository form of the active compound, such that a therapeutically effective amount of the compound reaches the target sites via systemic absorption and circulation.

For rectal administration, the compositions in the form of suppositories can be prepared by mixing the active ingredient with a suitable non-irritating excipient which is solid
30 at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the compound. Such excipients include cocoa butter and polyethylene glycols.

The active compounds may also be systemically administered to the platelet aggregation sites through absorption by the skin using transdermal patches or pads. The active compounds are absorbed into the bloodstream through the skin. Plasma concentration of the active compounds can be controlled by using patches containing different concentrations of active compounds.

One systemic method involves an aerosol suspension of respirable particles comprising the active compound, which the subject inhales. The active compound would be absorbed into the bloodstream via the lungs, and subsequently contact the target platelets in a pharmaceutically effective amount. The respirable particles may be liquid or solid, with a particle size sufficiently small to pass through the mouth and larynx upon inhalation; in general, particles ranging from about 1 to 10 microns, but more preferably 1-5 microns, in size are considered respirable.

Another method of systemically administering the active compounds to the platelet aggregation sites of the subject involves administering a liquid/liquid suspension in the form of eye drops or eye wash or nasal drops of a liquid formulation, or a nasal spray of respirable particles that the subject inhales. Liquid pharmaceutical compositions of the active compound for producing a nasal spray or nasal or eye drops may be prepared by combining the active compound with a suitable vehicle, such as sterile pyrogen free water or sterile saline by techniques known to those skilled in the art.

Intravitreal delivery may include single or multiple intravitreal injections, or via an implantable intravitreal device that releases P2Y₁₂ antagonists in a sustained capacity. Intravitreal delivery may also include delivery during surgical manipulations as either an adjunct to the intraocular irrigation solution or applied directly to the vitreous during the surgical procedure.

For systemic administration, plasma concentrations of active compounds delivered may vary according to compounds; but are generally 1×10^{-10} - 1×10^{-5} moles/liter, and preferably 1×10^{-8} - 1×10^{-6} moles/liter.

The pharmaceutical utility of P2Y₁₂ antagonist compounds of this invention is indicated by their inhibition of ADP-induced platelet aggregation. This widely used assay, as described in S.M.O. Hourani *et al. Br. J. Pharmacol.* 105, 453-457 (1992) relies on the measurement of the aggregation of a platelet suspension upon the addition of an aggregating agent such as ADP.

The present invention also provides novel compositions of matter. The compositions are pharmaceutically acceptable formulation comprising compounds of Formula I of high purity, and/or in a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier can be selected by those skilled in the art using conventional criteria. The pharmaceutically acceptable carrier include, but are not limited to, saline and aqueous electrolyte solutions, water polyethers such as polyethylene glycol, polyvinyls such as polyvinyl alcohol and povidone, cellulose derivatives such as methylcellulose and hydroxypropyl methylcellulose, petroleum derivatives such as mineral oil and white petrolatum, animal fats such as lanolin, polymers of acrylic acid such as carboxypolymethylene gel, vegetable fats such as peanut oil and polysaccharides such as dextrans, and glycosaminoglycans such as sodium hyaluronate and salts such as sodium chloride and potassium chloride.

Preferred compositions of the present invention comprises compounds of Formula Ib (mononucleotide), provided that both X_1 and X_2 are not O, when $n=1$, and X_1 is not O when $n=0$; and provided that X_2 is independently O, CH_2 , CHF, $CHCl$, CF_2 , CCl_2 when $Y' = H$; also provided that when $R_{10} = NH_2$ or O, and when R_5 and R_6 are taken together as oxygen doubly bonded to C, then R_7 is not equal to ortho-methylamino phenyl; further provided that when $n=p=1$, $X_2=CH_2$ and B' =adenosine, then R_1 and R_2 are not equal to naphthylenylmethyl, naphthylenylmethylene, or phenylmethylene.

Preferred compositions of the present invention also comprises compounds of Formula Ia, wherein B and B' are independently pyrimidine (pyrimidine/pyrimidine dinucleotide), provided that when $m+n+p = 1$, $R_{16} = CH_3$, and R_5 and R_6 are taken together as oxygen doubly bonded to C, then R_7 is not equal to CH_3 (Z' does not equal to acetate); also provided that when $m+n+p = 3$, B and B' = uridine, and R_5 and R_6 are taken together as oxygen doubly bonded to C, then R_7 is not equal to phenyl for $Y'=OR_1$ and 'or $Y= OR_4$ (Y and Y' does not equal to benzoyl); further provided that when $m+n+p = 1$, then both R_8 and R_9 are not CH_3 (Z' and Y' taken together do not equal isopropylidene).

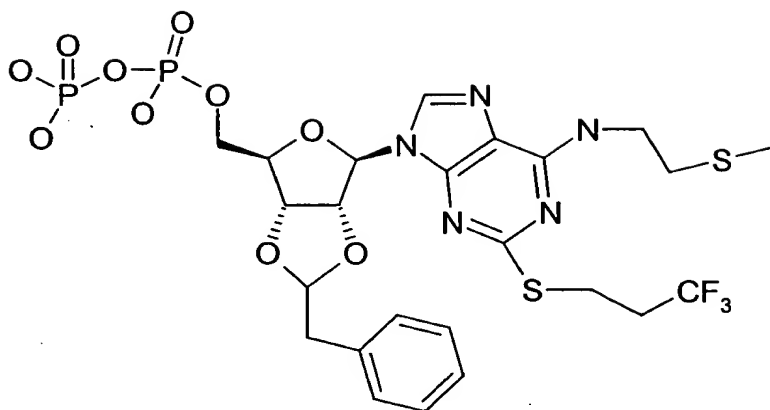
Preferred compositions of the present invention also comprises compounds of Formula Ia, wherein B is a purine or residue according to general formula IV, and B' is a pyrimidine residue according to general formula V, (purine/pyrimidine dinucleotide); provided that Y' is not equal to OCH_3 when Z' , Y, or $Y' = H$ or OH; further provided that R_8

is not equal to OCH_2CH_3 when $\text{R}_9 = \text{H}$ (Z' and Y' or Z and Y taken together do not equal to an orthoethylester).

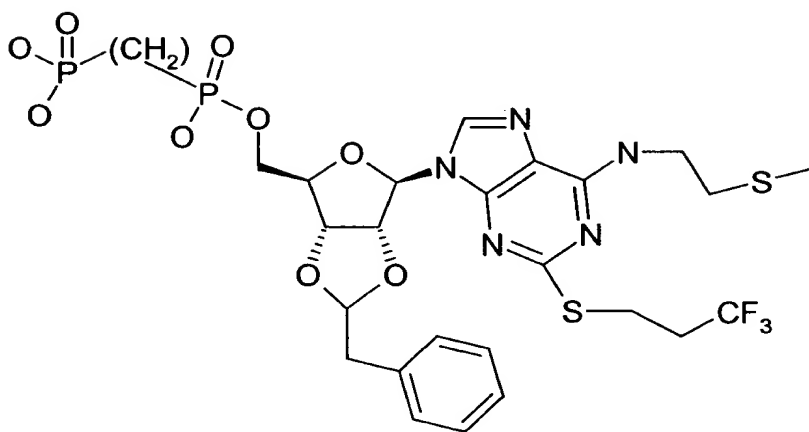
Preferred compositions of the present invention also comprises compounds of Formula Ia, wherein B and B' are independently a purine residue according to general formula IV, (purine/purine dinucleotide); provided that (a) Y or Y' is not equal to OCH_3 when $\text{R}_{10} = \text{NH}_2$ or O; (b) R_8 is not equal to OCH_3 or OCH_2CH_3 when $\text{R}_9 = \text{H}$; (c) both R_8 and R_9 are not equal to CH_3 ; (d) when $m+n+p = 1$, then R_8 and R_9 does not equal OCH_2CH_3 ; (e) when $\text{R}_{10} = \text{NH}_2$, and when R_5 and R_6 are taken together as oxygen doubly bonded to C, then R_7 is not equal to ortho-methylaminophenyl; (f) when $m+n+p = 1$, and when R_5 and R_6 are taken together as oxygen doubly bonded to C, then R_7 is not equal to $\text{CH}(\text{CH}_2\text{CH}_2\text{SCH}_3)\text{NHS}(\text{o-NO}_2\text{-Ph})$ or $\text{CH}(\text{CH}_2\text{Ph})\text{NHS}(\text{o-NO}_2\text{-Ph})$.

More preferred compositions of the present invention include the following compounds: 2' or 3' phenylcarbamate UTP, 2',3' di-phenylcarbamate UTP, 2',3' phenylacetaldehyde acetal ADP, di[3'(phenylcarbamate)dUp2dU], 2',3' phenylacetaldehyde acetal Up3U, di 2',3' phenylacetaldehyde acetal Up3U, 2',3' phenylacetaldehyde acetal Up4A, 2',3' phenylacetaldehyde acetal Ap4U, di 2',3' phenylacetaldehyde acetal Ap4U, 2',3' phenylacetaldehyde acetal Ip4U, 2',3' phenylacetaldehyde acetal Up4U, 2',3' phenylacetaldehyde acetal Ip4U, 2',3' phenylacetaldehyde acetal Up4dC, tetraphenylcarbamate Up4U, di 2',3' benzaldehyde acetal Ip4U, di 2',3' benzaldehyde acetal Up4U, 2',3' benzaldehyde acetal Up4U, di 2',3' phenylacetaldehyde acetal Cp4U, 2',3' phenylacetaldehyde acetal Cp4U, 2',3' phenylacetaldehyde acetal Up4C, 2',3' phenylacetaldehyde acetal Up4T, di 2',3' benzaldehyde acetal Cp4U, 2',3' benzaldehyde acetal Ip4U, 2',3' benzaldehyde acetal Up4U, 2',3' benzaldehyde acetal Up4dC, 2',3' benzaldehyde acetal Cp4U, 2',3' benzaldehyde acetal Up4C, 2',3' phenylpropionaldehyde acetal Up4U, di 2',3' phenylpropionaldehyde acetal Up4U, 2',3' benzaldehyde acetal Cp4C, Bis MANT Up4U, Mant Up4U, Di 2'/3' benzylacetal Up4U, Mono 2'/3' benzylacetal Up4U, Triphenyl carbamate Up4U, 2',3' phenylcarbamate Up4U, and monophenylcarbamate Up4U.

Preferred composition also comprises the following Compounds 1-21. In the following structures hydrogens which are understood to be present have been omitted for the sake of simplicity.

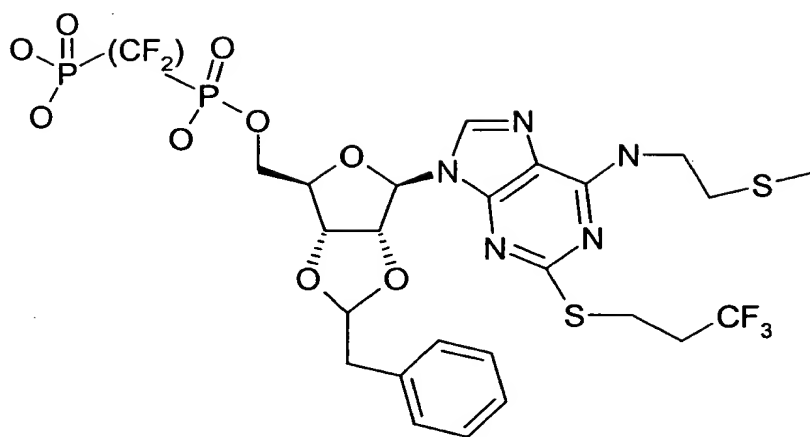


Compound 1



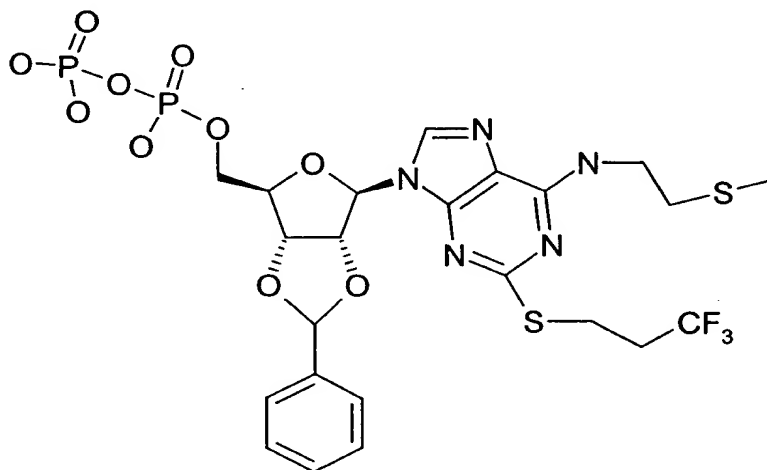
Compound 2

PubChem: 10034670

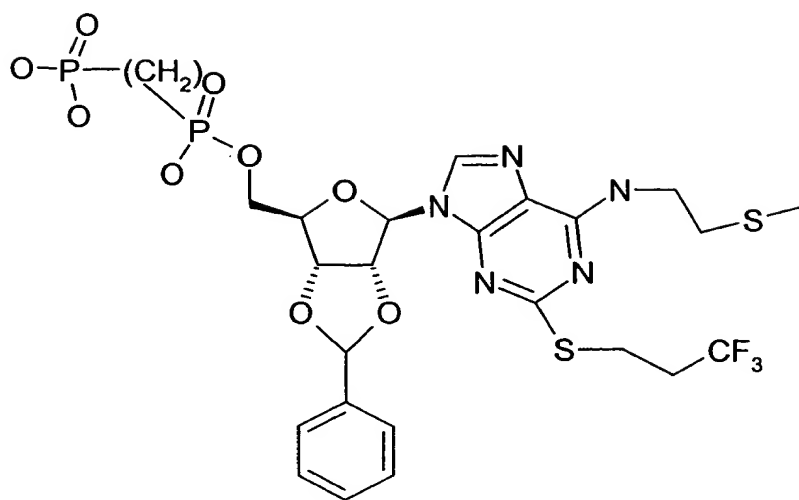


Compound 3

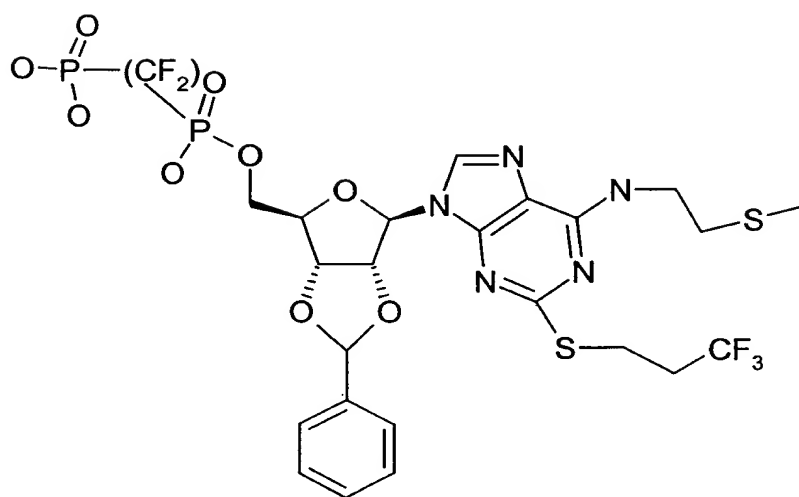
2-(3-trifluoromethylpropyl)thio-6-(2-methylthio) ethylamino-2',3'-(benzyl)methylenedioxy purine riboside 5'- α,β -difluoromethylene diphosphate



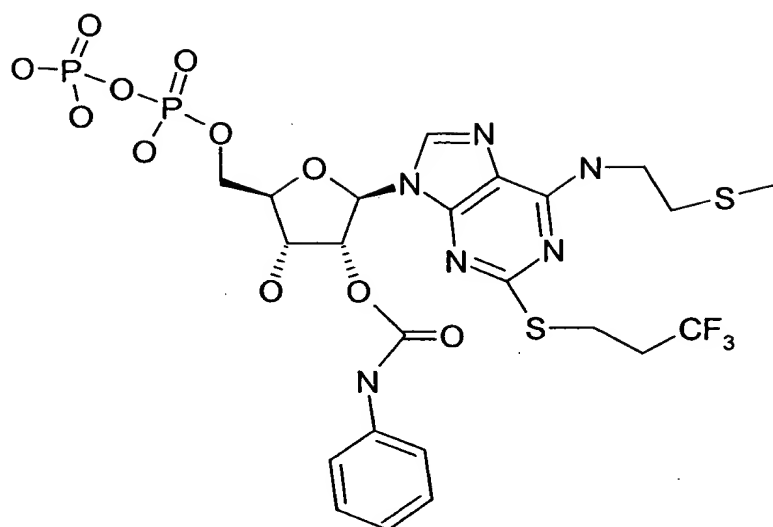
Compound 4



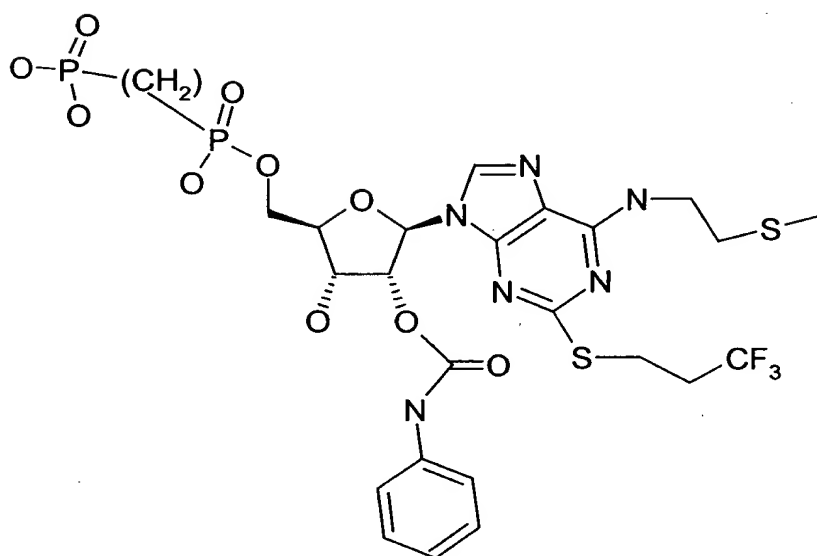
Compound 5



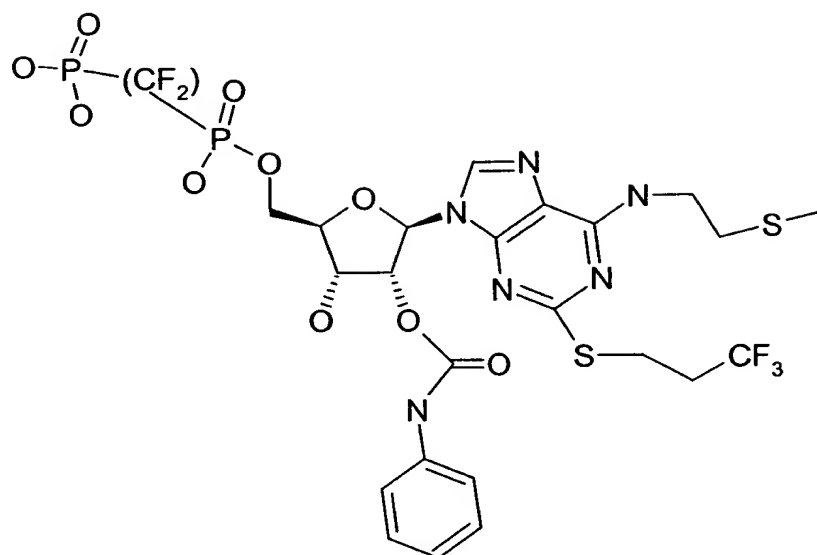
Compound 6



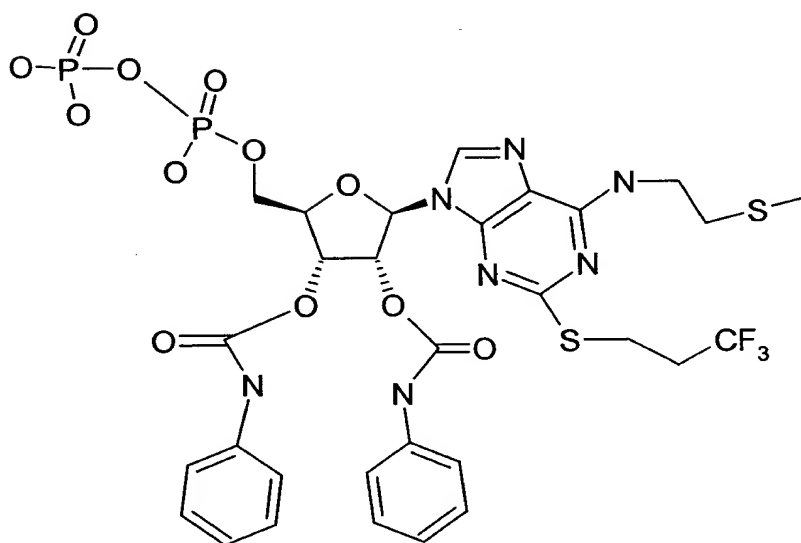
Compound 7



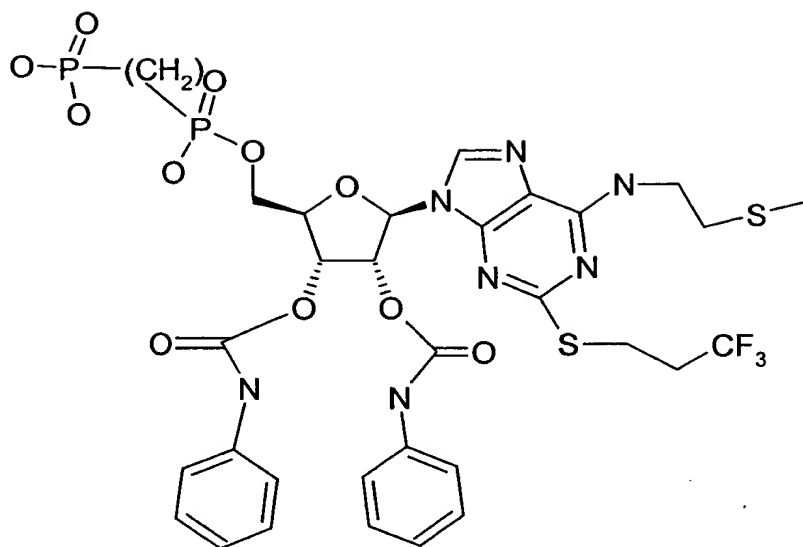
Compound 8



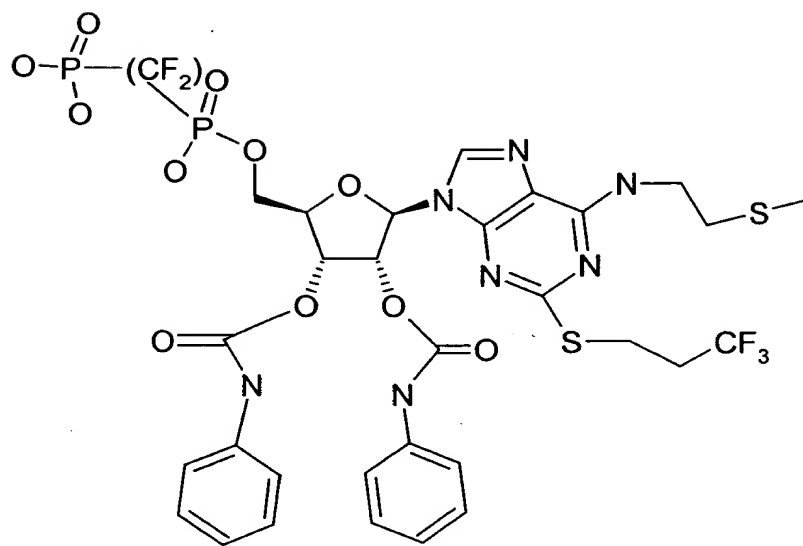
Compound 9



Compound 10

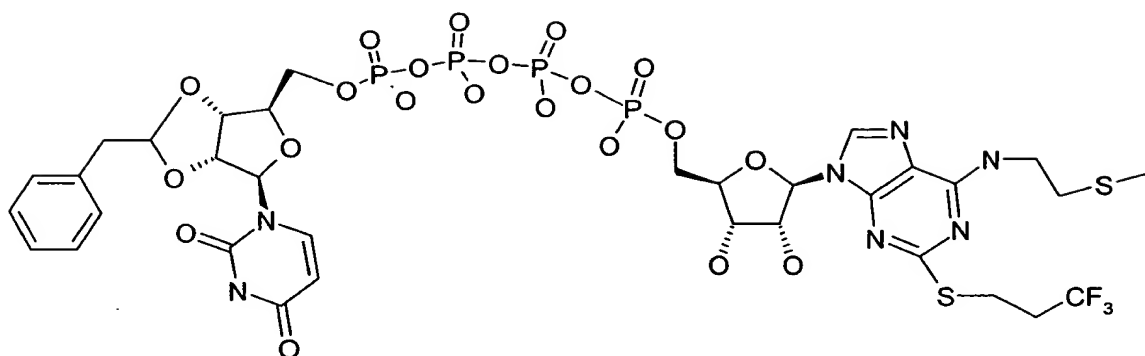


Compound 11

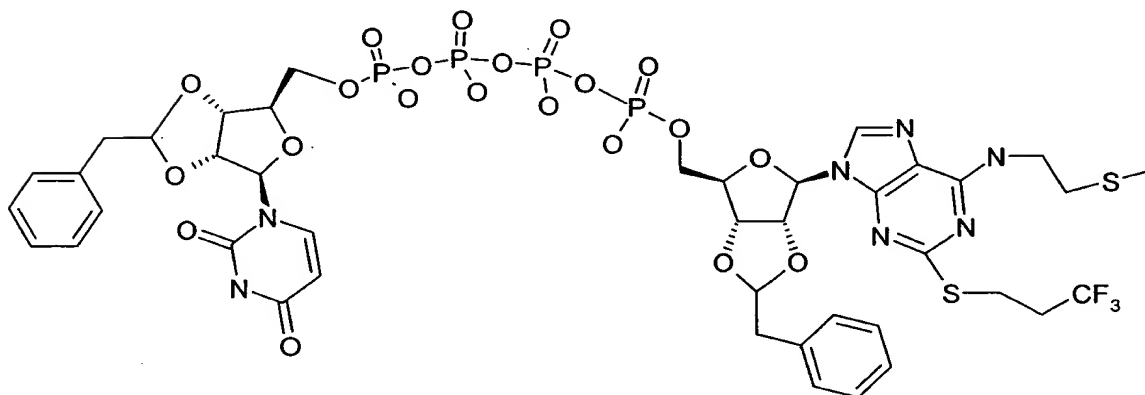


Compound 12

TOP SECRET

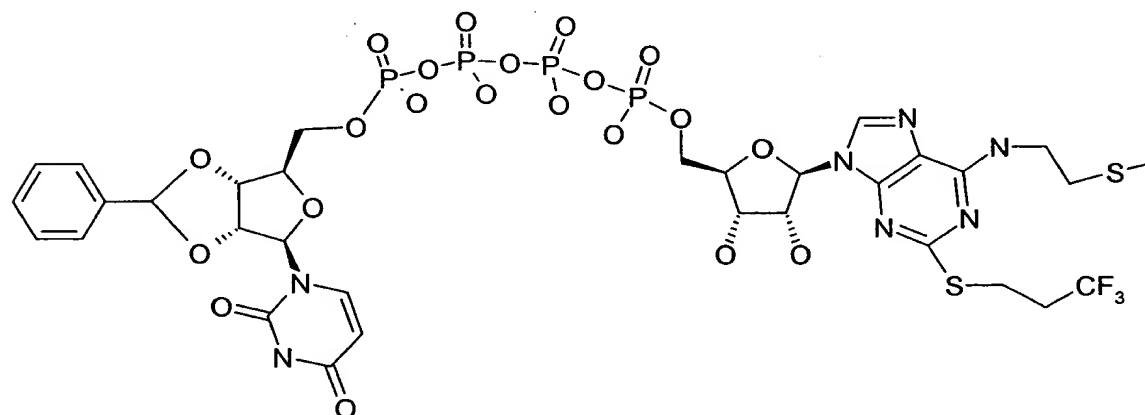


Compound 13

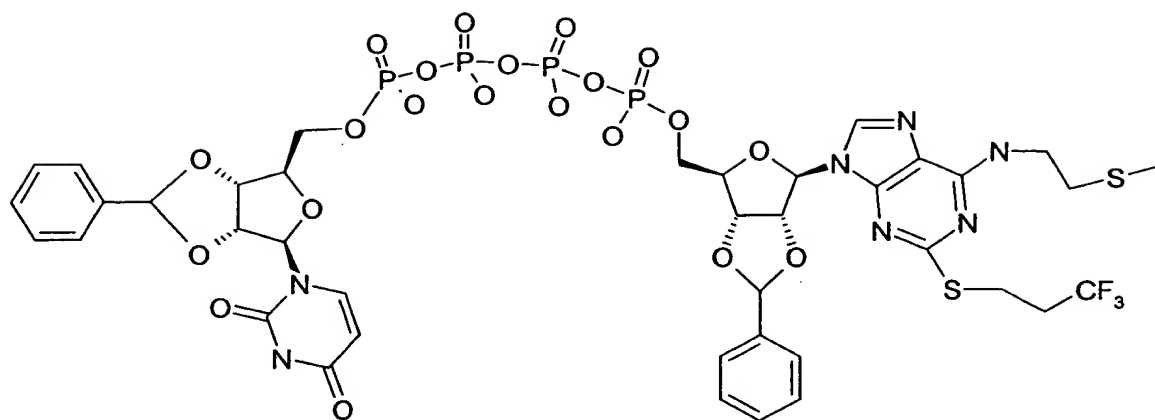


Compound 14

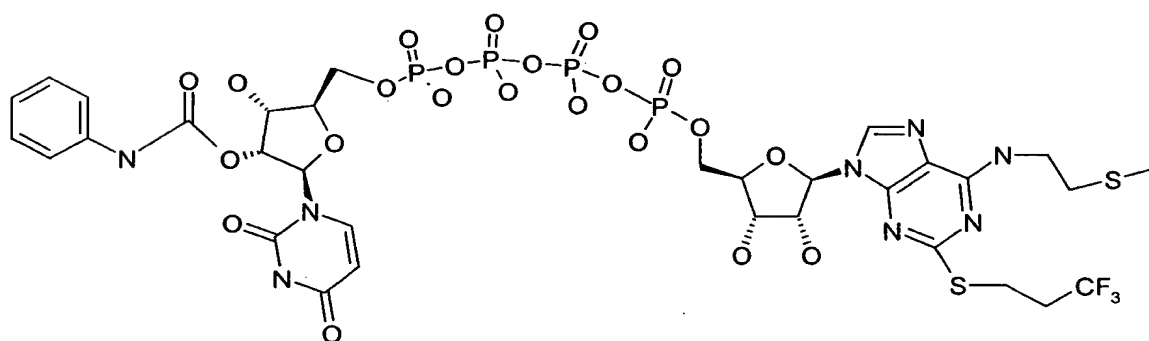
P¹-[2-(3-trifluoromethylpropyl)thio-6-(2-methylthio)ethylamino 2',3'-(benzyl)methylene dioxypurine riboside]-P⁴-(2',3'-(benzyl)methylene dioxypyrimidine uridine) tetraphosphate



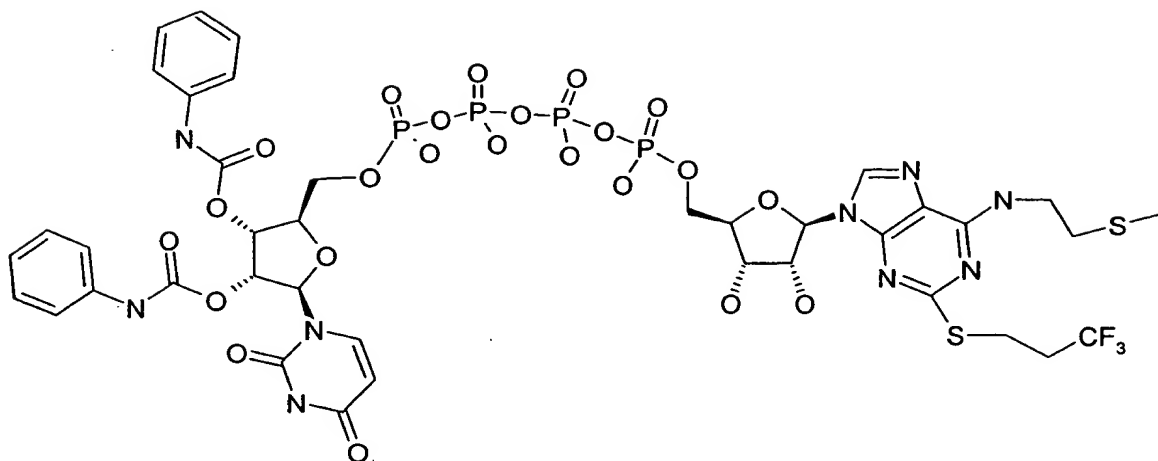
Compound 15



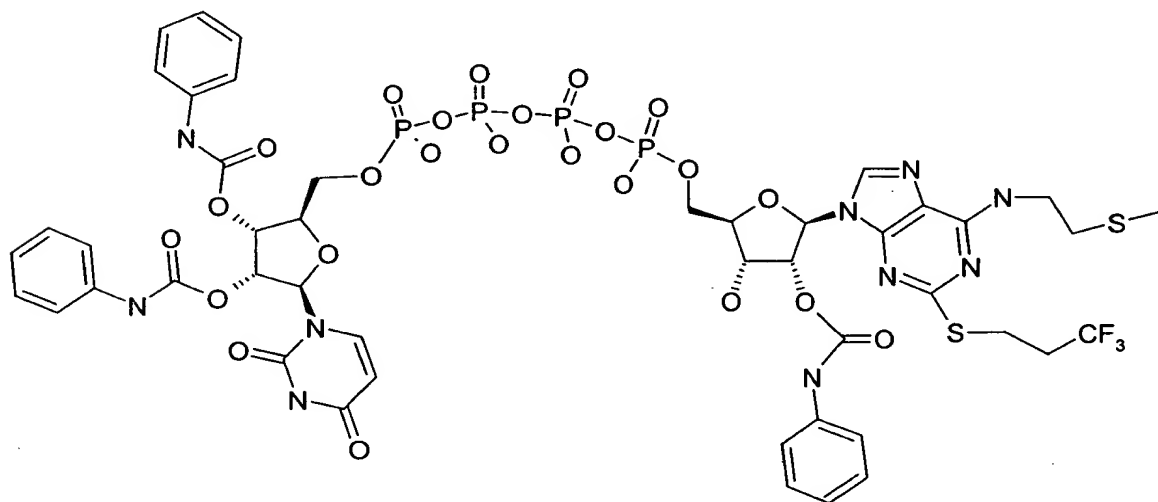
Compound 16



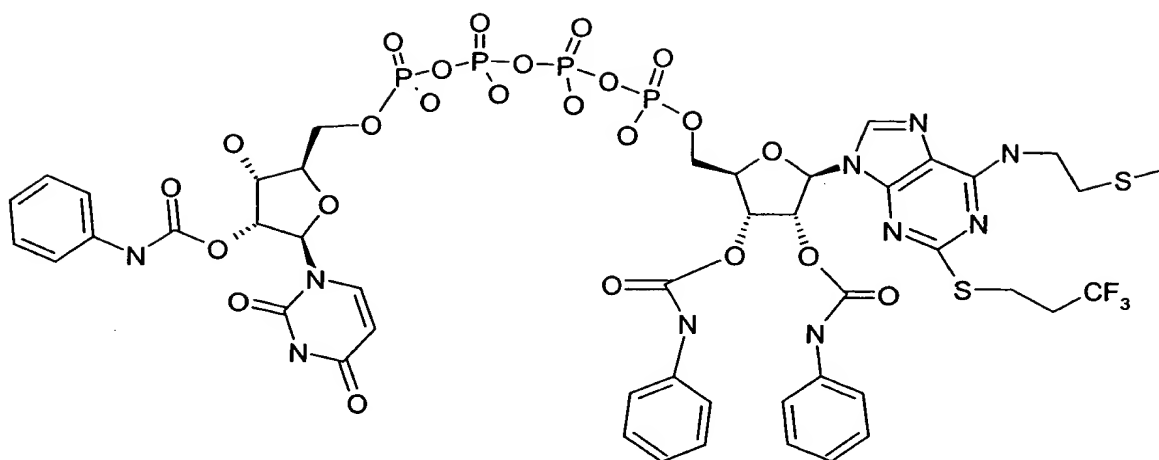
Compound 17



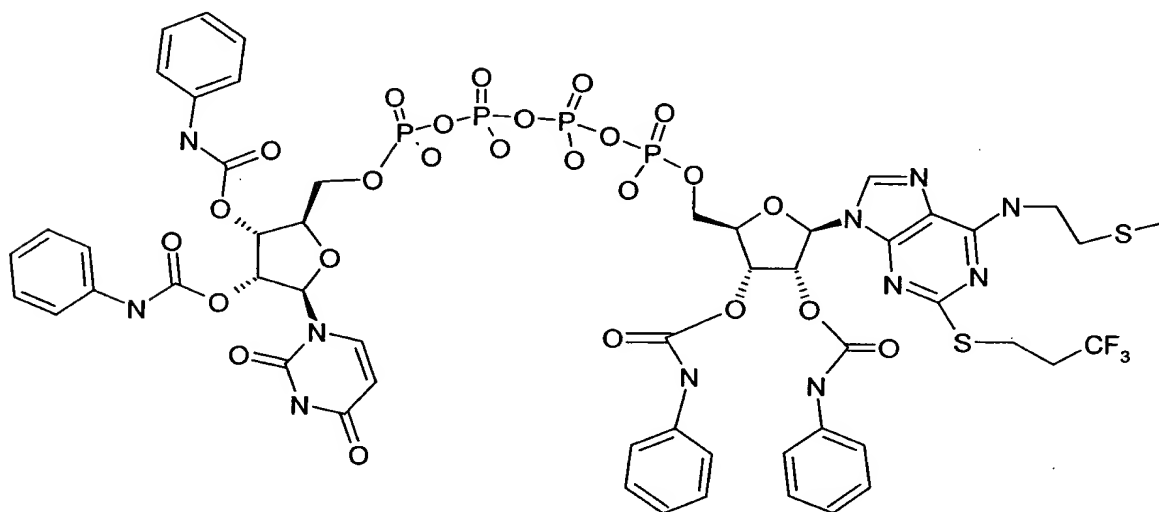
Compound 18



Compound 19



Compound 20



Compound 21

- 5 The invention is illustrated further by the following examples that are not to be construed as limiting the invention in scope to the specific procedures described in them.

EXAMPLES

Example 1

10 2'(3')-O-((phenylaminocarbonyl) -uridine 5'-)triphosphate

Uridine 5'- triphosphate, ditributylammonium salt (100 mg, 0.176 mmol; prepared from the trisodium salt by treatment with Dowex 50Wx4 H⁺ in water, followed by mixing the

protonated species with an excess of tributylamine, stripping and lyophilization) was dissolved in dry DMF (1 mL) and phenylisocyanate (19 μ L, 0.176 mmol) added. The reaction mixture was heated at 45°C for 15 minutes, at which point a further portion of phenylisocyanate (19 μ L, 0.176 mmol) was added. The solution was heated at 45°C

overnight and the DMF was removed on a rotary evaporator. The residual oil was partitioned between water (2 mL) and ethyl acetate (2 mL) and the layers were separated. The aqueous layer was extracted twice more with ethyl acetate (2 mL each) and the water was removed on a rotary evaporator. The residue was dissolved in water (1.5 mL) and the product isolated by repeated injections onto a preparative HPLC column (Alltech Nucleotide/Nucleoside C18, 7 μ m, 10 X 250 mm, gradient from 0.1 M ammonium acetate to methanol over 30 minutes, 5 mL/min, monitor at 260 nm). The yield of the carbamate was 26mg (22%, calculated for the tetraammonium salt). ¹H NMR showed the product to be a mixture of 2' and 3' carbamates. The product so obtained can be used for the purposes of this invention *per se* or can be activated with a suitable coupling agent (e.g. a carbodiimide) and reacted with a variety of nucleotides to generate novel dinucleoside polyphosphates.

¹H NMR (D₂O, 300 MHz): δ 4.10-4.47 (m, 4H), 5.17 (m, 1H), 5.83 (dd, 1H), 5.96 (m, 1H), 7.04 (t, 1H), 7.25 (m, 4H), 7.79 (m, 1H). ³¹P NMR (D₂O, 121.47 MHz): δ -9.54 (m, 1P), -10.20 (m, 1P), -21.87 (m, 1P).

Example 2

2'(3')-O-(phenylaminocarbonyl) -P¹,P⁴-di(uridine 5'-)tetraphosphate

[“monophenylcarbamate Up4U”], Di- 2'(3')-O-(phenylaminocarbonyl) -P¹,P⁴-di(uridine 5'-)tetraphosphate [“diphenylcarbamate Up4U”] and Tri- 2'(3')-O-(phenylaminocarbonyl) -P¹,P⁴-di(uridine 5'-)tetraphosphate [“triphenylcarbamate Up4U”]

P¹,P⁴-Di(uridine 5'-) tetraphosphate, ditributylammonium salt (211 mg, 0.182 mmol; prepared from the tetrasodium salt by treatment with Dowex 50Wx4 H⁺ in water, followed by mixing the protonated species with an excess of tributylamine, stripping and lyophilization) was dissolved in dry DMF (2 mL) and phenylisocyanate (40 μ L, 3.64 mmol) added in a single portion. The homogeneous reaction mixture was heated overnight at 45°C, whereupon TLC (silica gel, 50% isopropanol / 50% ammonium hydroxide) indicated a substantial conversion to two products. The solvent was removed on a rotary evaporator and

the residue was partitioned between water (7 mL) and ethyl acetate (10 mL). The layers were separated, and the aqueous was extracted twice more with ethyl acetate. (10 mL each). The water was removed from the aqueous extract and the residual oil lyophilized overnight. The solid obtained was reconstituted in water (3 mL) and the two products separated by repeated injections onto a semipreparative HPLC column (Alltech Nucleotide/Nucleoside C18, 7 μ m, 10 X 250 mm, gradient from 0.1 M ammonium acetate to methanol over 30 minutes, 5 mL/min, monitor at 260 nm). Stripping and lyophilization gave the mono-phenylcarbamate (48 mg, 27 % yield), di-phenylcarbamate (16 mg, 8%yield) and a trace amount of the triphenylcarbamate, as the tetraammonium salts. All three products were mixtures of the corresponding 2'/3' regioisomers.

Monophenylcarbamate: ^1H NMR (D_2O , 300 MHz): δ 4.08-4.65 (m, 9H), 5.14 (d, 1H), 5.75-5.94 (m, 4H), 7.01 (t, 1H), 7.22 (m, 4H), 7.76 (m, 2H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.17 (m, 2P), -21.81 (m, 2P).

Diphenylcarbamate: ^1H NMR (D_2O , 300 MHz): δ 4.13-4.43 (m, 8H), 5.12 (m, 2H), 5.84 (m, 4H), 7.01 (m, 2H), 7.21 (m, 8H), 7.75 (dd, 2H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.19 (m, 2P), -21.65 (m, 2P).

Triphenylcarbamate: ^1H NMR (D_2O , 300 MHz): δ 4.29 (m, 7H), 4.5.10 (m, 1H), 5.27 (m, 2H), 5.87 (m, 4H), 7.09 (m, 15H), 7.76 (d, 2H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.30 (m, 2P), -21.73 (m, 2P).

Example 3

P¹,P⁴-Tetra-(2'(3')-O-(phenylaminocarbonyl) di(uridine 5'-)tetraphosphate [tetraphenylcarbamate Up4U"]

This derivative was prepared according to the method of example 2. P¹,P⁴-Di(uridine 5'-) tetraphosphate, ditributylammonium salt (200 mg, 0.172 mmol) was treated with 16 eq of phenylisocyanate (300 μ L, 2.76 mmol) in DMF and stirred overnight at 35°C. The solvent was evaporated and the excess reagents removed by extraction of an aqueous solution of the product with ethyl acetate. Following preparative HPLC as previously described, 93 mg (30% yield) of the tetraphenylcarbamate was obtained.

Tetraphenylcarbamate ^1H NMR (D_2O , 300 MHz): δ 7.75 (d, 2H), 7.11 (m, 16H), 6.94 (m, 4H), 5.95 (d, 2H), 5.80 (d, 2H), 5.32 (m, 2H), 5.23 (m, 2H), 4.42 (m, 2H), 4.25 (m, 2H), 4.16 (m, 2H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.30 (m, 2P), -22.32 (m, 2P).

Example 4

2',3'-(benzyl)methylenedioxy-P¹,P⁴-di(uridine 5'-)tetraphosphate ["Mono 2'/3' benzylacetal Up4U"] and P¹,P⁴-Di-(2',3'-(benzyl)methylenedioxy) di(uridine 5'-)tetraphosphate ["Di 2'/3' benzylacetal Up4U"]

P¹,P⁴-Di(uridine 5'-) tetraphosphate, tetrasodium salt (290 mg, 0.332 mmol) was dissolved in 98% formic acid and phenylacetaldehyde, dimethyl acetal (110 uL, 0.662 mmol) added. The reaction was stirred at ambient temperature for 3 days, at which point TLC (silica gel, 50% isopropanol / 50% ammonium hydroxide) and HPLC (C18) showed good conversion to two less polar products. The formic acid was removed on a rotary evaporator, and the residue partitioned between 0.7 M ammonium bicarbonate (15 mL) and butyl acetate (15 mL). The layers were separated and the aqueous was washed with a further portion of butyl acetate (10 mL). The aqueous layer was stripped and the residue lyophilized overnight. The crude product was dissolved in water (5 mL) and the components separated by preparative HPLC (Waters Novapak C18, 6um, 25 X 100 mm, gradient from 0.1 M ammonium acetate to methanol over 30 minutes, 30 mL/min, monitor at 260 nm). The yield of the monoacetal was 88 mg (28%) and of the diacetal 60mg (17%), both as the tetraammonium salts.

Monoacetal: ¹H NMR (D₂O, 300 MHz): δ 2.99 (d, 2H), 4.01-4.32 (m, 8H), 4.77 (m, 2H), 5.33 (m, 2H), 5.74 (d, 1H), 5.81 (m, 2H), 7.21 (m, 5H), 7.64 (d, 1H), 7.79 (d, 1H). ³¹P NMR (D₂O, 121.47 MHz): δ -10.18 (m, 1P), -10.78 (m, 1P), -22.00 (m, 2P).

Diacetal: ¹H NMR (D₂O, 300 MHz): δ 2.98 (d, 4H), 3.99 (m, 4H), 4.27 (m, 2H), 5.27 (m, 2H), 5.36 (m, 2H), 5.73 (d, J= 8.1 Hz, 2H), 7.21 (m, 10H), 7.61 (d, J= 8.1 Hz, 2H). ³¹P NMR (D₂O, 121.47 MHz): δ -10.57 (m, 2P), -21.81 (m, 2P).

Example 5

2',3'-((benzyl)methylenedioxy) P¹,P³- uridine 5'-)triphosphate ["2'3' phenylacetaldehyde acetal Up3U"] and P¹,P³-Di-(2',3'-((benzyl)methylenedioxy) uridine 5'-)triphosphate ["di 2'3' phenylacetaldehyde acetal Up3U"]

P¹,P³-Di(uridine 5'-) triphosphate, trisodium salt (100 mg, 0.129 mmol) was dissolved in 98% formic acid and phenylacetaldehyde, dimethyl acetal (64 uL, 0.386 mmol) added. After overnight stirring at room temperature, the formic acid was removed, and the residue partitioned between 1 M sodium bicarbonate and ethyl acetate. Following removal of the

organic layer, the product was purified on preparative HPLC, as previously described. Following lyophilization, 40 mg (36%) of the monoacetal and 24 mg (19%) of the diacetal were obtained.

Monoacetal: ^1H NMR (D_2O , 300 MHz): δ 7.7s (d, 2H), 7.54 (d, 2H), 7.16 (s, 5H), 5.70 (m, 3H), 5.31 (s, 1H), 5.23 (s, 1H), 4.66 (m, 2H), 4.10 (m, 8H), 2.93 (d, 2H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.30 (m, 1P), 10.81 (m, 1P), -21.99 (m, 1P).

Diacetal: ^1H NMR (D_2O , 300 MHz): δ 7.51 (d, 2H), 7.15 (m, 10H), 5.65 (d, 2H), 5.31 (d, 2H), 5.20 (t, 2H), 4.63 (m, 2H), 4.13 (m, 2H), 3.88 (m, 4H), 2.90 (d, 4H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.75 (m, 2P), -21.97 (m, 1P).

Example 6

$\text{P}^1\text{-}2',3'\text{--}((\text{benzyl})\text{methylenedioxy}) (\text{uridine } 5')\text{-} \text{P}^4\text{--}(\text{deoxycytidine } 5')\text{-} \text{tetraphosphate}$ [$2'3'$ phenylacetaldehyde acetal Up4dC"]

$\text{P}^1\text{--}(\text{uridine } 5')\text{-} \text{P}^4\text{--}(\text{deoxycytidine } 5')\text{-} \text{tetraphosphate}$, tetrasodium salt (100 mg, 0.16 mmol) was dissolved in 98% formic acid (1 mL), and phenylacetaldehyde, dimethyl acetal (57 μL , 0.384 mmol) added. After overnight stirring, the formic acid was removed and the residue partitioned between 1 M sodium bicarbonate and ethyl acetate. After separation of the layers, the product was purified on preparative HPLC, as previously described. Yield 40 mg (36 %). This product was amenable to subsequent modification of the deoxy cytidine base by the procedures described in examples 9-13, giving rise to lipophilic bifunctional molecules falling within the scope of this invention.

Monoacetal: ^1H NMR (D_2O , 300 MHz): δ 7.98 (d, 1H), 7.62 (d, 1H), 7.21 (m, 5H), 6.11 (m, 2H), 5.74 (d, 1H), 5.39 (d, 1H), 5.31 (t, 1H), 4.77 (m, 2H), 4.45 (m, 1H), 4.32 (m, 1H), 4.03 (m, 5H), 2.99 (d, 2H), 2.29 and 2.21 (M, 2H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.15 (m, 1P), -10.68 (m, 1P), -21.98 (m, 2P).

Example 7

$3'\text{-O--}(\text{phenylaminocarbonyl})\text{-}2'\text{-deoxy} (\text{uridine } 5')\text{-} \text{monophosphate}$

Deoxyuridine 5'- monophosphate, tetrabutylammonium salt (135 mg , 0.274 mmol; prepared from the disodium salt by treatment with Dowex 50Wx4 H^+ , followed by stirring the resultant neutral species with excess tributylamine , stripping and lyophilization) was dissolved in dry DMF (1 mL). Phenylisocyanate (60 μL , 0.547 mmol) was added and the

mixture heated overnight at 45°C, at which time TLC (silica gel, 50% isopropanol / 50% ammonium hydroxide) and HPLC (C18) indicated a substantial conversion to a less polar product. The DMF was stripped on a rotary evaporator and the oily residue partitioned between water (10 mL) and ethyl acetate (10 mL). The layers were separated and the aqueous layer was rewashed with ethyl acetate (2 X 10 mL). The water was removed and the residue was dissolved in water (2 mL). The product was isolated by repeated injections onto semipreparative HPLC (Alltech Nucleotide/Nucleoside C18, 7µm, 10 X 250 mm, gradient from 0.1 M ammonium acetate to methanol over 30 minutes, 5 mL/min, monitor at 260 nm). The yield was 67 mg as the diammonium salt (53 %).

¹H NMR (D₂O, 300 MHz): δ 2.21 (m, 2H), 3.84 (s, 2H), 4.13 (s, 1H), 5.08 (d, 1H), 5.63 (d, 1H), 6.06 (t, 1H), 6.89 (br. t, 1H), 7.10 (m, 4H), 7.72 (d, 1H).

³¹P NMR (D₂O, 121.47 MHz): δ -2.31 (s).

P¹-(3'-O-(phenylaminocarbonyl)-2'-deoxyuridine 5'-)-P⁴-(uridine 5'-)tetraphosphate

Uridine 5'-triphosphate, ditributylammonium salt (prepared from the trisodium salt by treatment with Dowex 50Wx4 H⁺, followed by stirring the resultant neutral species with excess tributylamine, stripping and lyophilization) is treated with 1.5 equivalents of dicyclohexylcarbodiimide in DMF for 2 hours at room temperature. The dicyclohexylurea is filtered off, and the resultant uridine 5'-cyclical triphosphate is treated with 3'-O-(phenylaminocarbonyl)-2'-deoxy (uridine 5')-monophosphate, which is in the monotributylammonium salt form. The reaction mixture is stirred for several days at 45°C, and the solvent is removed. The products are separated by preparative HPLC, as has been previously described.

Example 8

2'(3')-(2-methylamino)benzoyl-P¹,P⁴-di(uridine 5'-)tetraphosphate ("MANT Up4U") and P¹,P⁴-Di-(2'(3')-(2-methylamino)benzoyl uridine 5'-)tetraphosphate ("Bis MANT Up4U")

P¹,P⁴-Di(uridine 5'-)tetraphosphate, tetrasodium salt (800 mg, 0.93 mmol) was dissolved in water (5 mL) and the pH adjusted to 7.6 by the addition of solid sodium bicarbonate. N,N-dimethylformamide (DMF, 5 mL) was added, followed by N-methylisatoic anhydride (231 mg, 1.3 mmol) and the suspension was heated at 50°C for 2.5 hrs. TLC (silica gel, 50% isopropanol, 50% ammonium hydroxide) indicated that the reaction was not done by

this time, so a further portion of N-methylisatoic anhydride (100 mg, 0.56 mmol) was added and the reaction heated for another hour. The DMF was removed on a rotary evaporator and the residue was dissolved in a minimum of water and applied to a DEAE Sephadex A-25 column (3 X 60 cm). The column was eluted with a stepwise gradient from water to 1 M ammonium bicarbonate and the eluent monitored with a UV detector set at 254 nm. The two products that eluted were collected separately and the solvent was removed from each and the residue lyophilized overnight. ¹H NMR indicated that the first product to elute was the monoacylated compound, while the latter was the diacylated derivative, and that both were mixtures with the acylation at either the 2' or 3' hydroxyls, but without two carbamates on the same sugar. The yield of the monoaminobenzoylated product was 150 mg (16%); the yield of the diaminobenzoylated compound was 91 mg (8.7%).

Monoaminobenzoylated derivative: ¹H NMR (D₂O, 300MHz): δ 2.70 (s, 3H), 4.09-4.55(m, 9H), 5.34 (m, 1H), 5.71 (m, 2H), 5.83 (dd, 1H), 6.01 (m, 1H), 6.57 (m, 1H), 6.65 (m, 1H), 7.25 (t, 1H), 7.72 (d, 2H), 7.81 (m, 2H). ³¹P NMR (D₂O, 121.47 MHz): δ -10.20 (m, 2P), -21.83 (m, 2P).

Diaminobenzoylated derivative: ¹H NMR (D₂O, 300 MHz): δ 2.69 (s, 6H), 4.15-4.51 (m, 8H), 5.27 (m, 2H), 5.86 (m, 4H), 6.60 (m, 4H), 7.30 (m, 2H), 7.79 (m, 4H). ³¹P NMR (D₂O, 121.47 MHz): δ -10.16 (m, 2P), -21.76 (m, 2P).

Example 9

P¹-(4-N-(4-methoxyphenyl)aminocarbonylcytidine 5'-) -P⁴-(uridine 5'-) tetrphosphate

P¹-(cytidine 5'-) -P⁴-(uridine 5'-) tetrphosphate, ditributylammonium salt (50 mg, 0.043mmol; prepared from the tetraammonium salt by treatment with Dowex 50Wx4 H⁺ in water, followed by mixing the protonated species with an excess of tributylamine in methanol, stripping and lyophilization) was dissolved in dry DMF (1mL) and tributylamine (10 uL, 0.43 mmol), and p-methoxyphenylisocyanate (8.4 uL, 0.648 mmol) were added in a single portion. The homogeneous reaction mixture was heated overnight at 35°C, whereupon TLC (silica gel, 50% isopropanol / 50% ammonium hydroxide) and HPLC (C18) indicated a substantial conversion to a single product. The solvent was removed on a rotary evaporator and the residue dissolved in water (1mL). The product was isolated by repeated injections onto a semi-preparative HPLC column (Alltech Nucleotide/Nucleoside C18, 7um, 10 X 250 mm, gradient from 0.1 M ammonium acetate to methanol over 30 minutes, 5 mL/min,

monitor at 260 nm). Stripping and lyophilization gave the p-methoxyphenylurea (24 mg, 55 % yield), as the tetraammonium salt.

The product so obtained can be derivatized on the 2' and/or 3' hydroxyl groups according to the foregoing methods (e.g. Examples 2-6).

- 5 ^1H NMR (D_2O , 300 MHz): δ 3.59 (s, 3H), 4.01-4.20 (m, 10H), 5.68 (m, 3H), 6.19 (d, 1H), 6.71 (d, 2H), 7.18 (d, 2H), 7.67 (d, 1H), 8.06 (d, 1H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.13 (m, 2P), -21.76 (m, 2P).

Example 10

10 **P^1 -((4-bromophenyl)ethenocytidine 5'-)- P^4 -(uridine 5'-) tetraphosphate**

P^1 -(cytidine 5'-)- P^4 -(uridine 5'-) tetraphosphate, tetrasodium salt (500 mg, 0.57 mmol) was dissolved in water (5 mL) and a solution of 2,4'-dibromoacetophenone (792 mg, 2.85 mmol) in DMF (15 mL) added. The mixture was heated overnight at 40°C, and a further portion of the dibromoketone (400 mg, 1.44 mmol) in DMF (5 mL) added. The rection was heated a further 5 hrs, and the solvents removed by evaporation. The residue was partitioned between water (20 mL) and ethyl acetate (25 mL) and the layers separated. The aqueous layer was washed with further ethyl acetate (2x15 mL) and the aqueous evaporated to dryness. The residue was dissolved in water (5 mL) and the product was isolated by repeated injections onto a semi-preparative HPLC column (see example 6 for conditions). The yield of the pure etheno compound was 80 mg (13.5%)

20 ^1H NMR (D_2O , 300 MHz): δ 4.06 (m, 8H), 4.36 (m, 2H), 5.64 (dd, 2H), 6.07 (d, 1H), 6.74 (d, 1H), 7.45 (d, 2H), 7.54 (d, 2H), 7.59 (d, 1H), 7.63 (d, 1H), 7.93 (s, 1H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.09 (m, 2P), -21.59 (m, 2P).

Example 11

25 **P^1 -((4-bromophenyl)etheno-2'-deoxycytidine 5'-)- P^4 -(uridine 5'-) tetraphosphate**

Example 8 product was prepared from 100 mg P^1 -(2'-deoxycytidine 5'-)- P^4 -(uridine 5'-) tetraphosphate, tetrasodium salt and 2,4'-dibromoacetophenone, according to the general method of example 7. Yield= 35 mg (30%).

- 30 ^1H NMR (D_2O , 300 MHz): δ 2.31 (m, 2H), 4.03 (m, 8H), 5.60 (dd, 2H), 6.41 (t, 1H), 6.73 (d, 1H), 7.53 (m, 5H), 7.65 (d, 1H), 7.93 (s, 1H). ^{31}P NMR (D_2O , 121.47 MHz): δ -10.11 (m, 2P), -21.58 (m, 2P).

Example 12

P¹, P⁴-Di((4-bromophenyl)ethenocytidine 5'-) - tetraphosphate

Example 9 product was prepared from 50 mg P¹,P⁴-Di(cytidine 5'-) tetraphosphate, tetrasodium salt and 2,4'-dibromoacetophenone, according to the general method of example 7. Yield= 20 mg (29%).

¹H NMR (D₂O, 300 MHz): δ 4.24 (m, 10H), 5.98 (d, 2H), 6.39 (d, 2H), 7.14 (m, 8H), 7.45 (m, 4H). ³¹P NMR (D₂O, 121.47 MHz): δ -10.13 (m, 2P), -21.68 (m, 2P).

Example 13

P¹-((4-phenylphenyl)ethenocytidine 5'-) -P⁴-(cytidine 5'-) tetraphosphate

Example 10 was product prepared from 50 mg P¹,P⁴-Di(cytidine 5'-) tetraphosphate, tetrasodium salt and 2-bromo-4'-phenylacetophenone, according to the general method of example 7. Yield= 15 mg (13%).

¹H NMR (D₂O, 300 MHz): δ 4.10 (m, 10H), 5.48 (d, 1H), 5.87 (m, 2H), 6.68 (d, 1H), 7.20 (m, 3H), 7.36 (m, 6H), 7.68 (m, 3H). ³¹P NMR (D₂O, 121.47 MHz): δ -10.08 (m, 2P), -21.78 (m, 2P).

The products of examples 7-10 can be further derivatized according to the methods of Examples 2-6, to give bifunctional molecules that fall within the scope of the invention.

Example 14

Inhibition of ADP-Induced Platelet Aggregation

Isolation of Platelets: Human blood was obtained from informed healthy volunteers. Blood was collected into one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g citric acid, and 2.5 g glucose in 100 ml dH₂O). Blood was centrifuged at 800 x g for 15 min at room temperature and the platelet-rich plasma removed and incubated for 60 min at 37 °C in the presence of 1 mM acetylsalicylic acid followed by centrifugation at 1000 x g for 10 min at room temperature. The platelet pellet was resuspended at a density of 2 x 10⁸ cells/ml with HEPES-buffered Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 5 mM glucose, 10 mM HEPES pH 7.4, 0.2% bovine serum albumin, and 0.05 U/ml apyrase).

Aggregation Studies: ADP-induced platelet aggregation was determined by measuring the transmission of light through a 0.5 ml suspension of stirred (900 rpm) aspirin-treated washed platelets in a lumi-aggregometer at 37 °C (Chrono-Log Corp. Havertown, PA). The baseline of the instrument was set using 0.5 ml of Hepes-buffered Tyrode's solution. Prior to aggregation measurements, the platelet suspension was supplemented with 2 mM CaCl₂ and 1 mg/ml fibrinogen. Platelet aggregation was initiated by the addition of indicated concentrations of ADP or other agonists, and the light transmission continuously recorded for at least 8 min. When inhibitors of platelet aggregation were tested, platelets were incubated for 3-6 min in the presence of indicated concentrations of inhibitor before addition of ADP or other agonists, and the response recorded for at least 8 min. The potency of agonists and inhibitors of platelet aggregation was calculated from both, the rate of aggregation and the maximal extent of aggregation obtained for each determination by fitting the data to a four-parameter logistic equation using the GraphPad software package (GraphPad Corp. San Diego, CA).

The ability of P2Y₁₂ antagonists to inhibit platelet aggregation is presented in this application as the percent inhibition of the aggregation induced by a maximally effective concentration of ADP. When a broad range of concentrations of P2Y₁₂ antagonist was tested (usually from 1nM to 100 µM), an IC₅₀ value was also obtained. IC₅₀ values represent the concentration of antagonist needed to inhibit by 50% the aggregation elicited by a given concentration of ADP.

Example 15

Effects on Platelet Aggregation *In Vivo*

To evaluate the ability of these compounds to inhibit platelet aggregation *in vivo*, an experimental protocol similar to the method of R. G. Humphries et al. (Br. J. Pharmacol. 115:1110-1116, 1995) will be performed.

Surgical Preparation and Instrumentation: Male Sprague-Dawley rats are anesthetized. Body temperature is maintained at 37 ± 0.5°C with a heating lamp. Animals breathe spontaneously and a tracheotomy is performed to ensure a patent airway. A cannula containing heparinized saline is introduced into the left femoral artery and connected to a transducer to record blood pressure and heart rate. Cannulae containing non-heparinized

saline are introduced into the left common carotid artery and left jugular vein for withdrawal of arterial blood samples and i.v. administration of compounds, respectively.

Experimental Protocol: Either compound or vehicle is administered to each animal as an infusion. Blood samples are taken immediately prior to the first infusion, at the end of each infusion and 20 min after cessation of the final infusion for measurement of platelet aggregation *ex vivo*. Immediately after sampling, ADP-induced platelet aggregation is measured in duplicate in 0.5 ml blood samples diluted 1:1 with saline and incubated at 37°C for 4 min. For the final minute of this period, cuvettes are transferred to a lumi-aggregometer and the sample stirred at 900 rpm. ADP (3 µM) is added in a volume of 20 µl and the aggregation response is recorded.

Example 16

Inhibition of Thrombus Formation in Anesthetized Rats

To evaluate the effect of these compounds on thrombus formation *in vivo*, the following experimental protocol will be performed.

Rats (CD-1; male; approximately 350 grams; Charles River, Raleigh, NC), are anesthetized with sodium pentobarbital (70 mg/kg i.p.). The abdomens are shaved and a 22 gauge intravenous catheter is inserted into a lateral tail vein. A midline incision is made and the intestines are wrapped in saline-soaked gauze and positioned so the abdominal aorta is accessible. The inferior vena cava and abdominal aorta are carefully isolated and a section (approx. 1 cm) of the abdominal aorta (distal to the renal arteries proximal to the bifurcation) is dissected. All branches from the aorta in this section are ligated with 4-0 silk suture. A 2.5 mm diameter flow probe connected to a Transonic flow meter is placed on the artery and a baseline (pre-stenosis) flow is recorded. Two clips are placed around the artery decreasing the vessel diameter by approximately 80%. A second baseline flow measurement is taken (post-stenosis) and the hyperemic response is tested. Animals are then treated with either compound or saline i.v., via tail vein catheter. Thrombosis is induced five minutes after treatment by repeated external compressions of the vessel with hemostatic forceps. Two minutes post-injury, the vessel compressions are repeated and a 10 minute period of flow monitoring is started. Animals are monitored continuously for a minimum of the first ten minutes post-injury. After twenty minutes (post-injury), a flow measurement is repeated and

the animals are euthanized. The section of the aorta that includes the injured section is harvested and placed in 10% formalin for possible histologic evaluation.

Example 17

5 **Inhibition of Thrombus Formation in Anesthetized Dogs**

To evaluate the effect of these compounds on dynamic thrombus formation *in vivo*, the following experimental protocol similar to the method of J. L. Romson et al. (*Thromb. Res.* 17:841-853, 1980) will be performed.

10 *Surgical Preparation and Instrumentation:* Briefly, purpose-bred dogs are anesthetized, intubated and ventilated with room air. The heart is exposed by a left thoracotomy in the fifth intercostal space and suspended in a pericardial cradle. A 2-3 cm segment of the left circumflex coronary artery (LCCA) is isolated by blunt dissection. The artery is instrumented from proximal to distal with a flow probe, a stimulation electrode, and a Goldblatt clamp. The flow probe monitors the mean and phasic LCCA blood flow velocities. The stimulation electrode and its placement in the LCCA and the methodology to induce an occlusive coronary thrombus have been described previously (J. K. Mickelson et al., *Circulation* 81:617-627, 1990; R. J. Shebuski et al., *Circulation* 82:169-177, 1990; J. F. Tschopp et al., *Coron. Artery Dis.* 4:809-817, 1993).

15 *Experimental Protocol:* Dogs are randomized to one of four treatment protocols (n=6 per treatment group) in which the control group receives saline i.v. and the three drug-treated groups are administered compound i.v. Upon stabilization from the surgical interventions, dogs receive either saline or compound. After approximately 30 minutes, an anodal current is applied to the LCCA for 180 min. The number and frequency of cyclic flow variations (CFV) that precede formation of an occlusive thrombus are recorded. These cyclic phenomena are caused by platelet thrombi that form in the narrowed lumen as a result of platelet aggregation (J. D. Folts et al., *Circulation* 54:365-370, 1976; Bush et al., *Circulation* 69:1161-1170, 1984). Zero flow in the LCCA for a minimum of 30 minutes indicates a lack of antithrombotic efficacy (L.G. Frederick et al., *Circulation* 93:129-134, 1996).

Example 18

30 **ADP-Induced Aggregation of Different Compounds**

Different compounds were tested for their inhibition of ADP-induced aggregation and their IC₅₀ according to the protocols in Example 14; the results are shown in Figure 1. The

bar graphs in the figure illustrate the effect of 100 μ M concentration of the compound on ADP-induced platelet aggregation, and the data are expressed as % inhibition of the ADP response.

Figure 1 shows the structure and abbreviated name of each compound and its activity.

- 5 Where hydrogens are understood to be present, they have been omitted for the sake of simplicity. For example, for the first structure of the figure, it is implied that there are hydrogens at the 3- position of the pyrimidine ring, at the 3' position of the ribose on the oxygen, and on the nitrogen of the carbamate at the 2' position of the ribose. In addition, as disclosed within the scope of the present invention, it is implied that the oxygens that are not
- 10 doubly bonded to the phosphorous atoms are either present in the ionized form as salts with a counterion, or are bonded to a hydrogen atom. For simplicity, some of the structures in the figure are portrayed in the salt form, but this should not be interpreted as excluding the possibility that hydrogens could be present instead.

Several parent compounds, Up4U, Ip4U, Up3U, and Cp4U, without modifications on the furanose hydroxyl groups, have been included at the end of the figure to illustrate the utility of the present invention. However, these unmodified parent compounds do not inhibit the ADP-induced aggregation and are not within the scope of the present invention.